Horizontally Transferable Elements among Enterococci

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von Jenny A. Laverde Gomez
aus Bogota, Kolumbien

1. Referent: Prof. Dr. Dieter Jahn
2. Referent: Privatdozent Dr. Guido Werner
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- Chromosomal Transfer of the *Enterococcus faecalis* Pathogenicity Island. Laverde Gómez J., Fleige,C., Werner,G.
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A mis padres Abelardo y Lucila,
mi inspiracion y modelo de vida.

For we know partially and we prophesy partially
1 Corinthians 13:9

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List of Abbreviations

bp	Base pair
CFU/ml	Colony forming units per mililiter
esp	Enterococcal surface protein gene
℃	Centigrades
h	Hour
hyl_{Efm}	putative hyaluronidase of *Enterococcus faecium*
kb	Kilobase pairs
M	Molar (mol per liter)
mg/l	Miligram per Liter
min.	Minutes
mM	Milimolar (milimol per liter)
µl	Microgram per mililiter
µM	Micromolar (µmol per liter)
nmol	Nanomol
N	Normal (equivalent mol per liter)
pmol	Picomol
rpm	Revolutions per minute
s	Seconds
µg	Microgram
µg/ml	Microgram per mililiter
AFLP	Amplified Fragment Length Polymorphism
APH	Phosphotransferases
BLAST	Basic Local Alignment Search Tool
CC	Clonal complex
CDS	Coding sequence
CLI	Clindamycin
Da	Dalton
DNA	Desoxirribonucleic acid
DR	Direct repeat
ERY	Erythromycin
Esp	Enterococcal surface protein
FACS	Fluorescence-activated cell sorting
FUS	Fusidic acid

GEN	Gentamycin
GI	Genomic island
HLR	High level resistance
ICE	Integrative Conjugative Element
IS	Insertion sequence
kDa	Kilodalton
LIN	Lincosamide
MDR	Multi drug resistant
MLS	Macrolide, Lincosamide, Streptogramine B group
MLST	Multilocus Sequence Typing
MLVA	Multiple Loci Variable number of tandem repeats analysis
ng	Nanogram
nM	Nanomolar
ORF	Open reading frame
OTE	Oxitetracycline
PAI	Pathogenicity island
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PEN	Penicillin
PFGE	Pulse field gel electrophoresis
PTS	Phosphotransferase system
PTS	Phosphotransferase system
RAM	Rifampicin
RNA	Ribonucleic acid
SPE	Spectinomycin
SSBP	ssDNA binding protein
ssDNA	Single stranded DNA
ST	Sequence type
STR	Streptomycin
STR	Streptomycin
T	Transconjugants
T4SS	Type four secretion system
TA	Toxin-antitoxin system
TEM	Transmission electron microscopy
Tn	Transposon
UTI	Urinary tract infection
VAN	Vancomycin
VNTR	Multiple Loci Variable number of tandem repeats

Abstract

Enterococci (mainly *Enterococcus faecalis* and *E. faecium*) are the third leading cause of hospital associated infections and have gained increased importance due to their fast adaptation to the clinical environment. Hospital associated clonal types of *E. faecium* and *E. faecalis* constitute subgroups within their own species that show a tendency to spread and persist among the nosocomial setting, to acquire resistance treats and may express increased pathogenicity. The high recombination rates of enterococci are associated to the presence of several mobile elements encoding antibiotic resistance and pathogenicity traits.

This work presents results of the analysis of two virulence-associated mobilizable elements of Enterococcus: the putative hyaluronidase gene of *E. faecium* hyl_{Efm}, and the enterococcal surface protein *esp*-containing pathogenicity island (PAI) of *E. faecalis*. The hyl_{Efm} gene, contained within a putative genomic island (GI), is enriched among the polyclonal subpopulation of hospital associated *E. faecium* strains. The hyl_{Efm} GI (17,824 bp) was found to be conserved among 39 hyl_{Efm} positive strains with variation in a specific region downstream of hyl_{Efm} (n=18) and was located on large plasmids (150 to 350 kb) (n=37). hyl_{Efm}-*vanA* plasmids were horizontally transferred into three different *E. faecium* recipient strains but not into *E. faecalis*. Sequencing of pLG1 resolved putative plasmid replication, conjugation and maintenance determinants, as well as additional putative pathogenicity factors (e.g. *pilA*) and antibiotic resistance genes (i.e. *vanA*, *erm*(B)). Although the *E. faecalis* PAI (153 kb) is thought to be mobile, so far only internal fragments of the PAI or larger chromosomal regions containing it had been mobilized. This work presents the horizontal transfer of the entire PAI element from the chromosome of *E. faecalis* strain UW3114 (ca. 200 kb) when selecting for transfer of erythromycin resistance (located in plasmid pLG2). The PAI excised and circularized precisely, and transferred horizontally integrating site-specifically into the chromosome of *E. faecalis* (intergenic region) and *E. faecium* (tRNAlys). Several phenotypic characteristics accompanied the acquisition of the PAI: Esp on the cell surface, enhanced biofilm formation and cytolytic activity in *E. faecalis*, although no differences were seen in pathogenicity using mouse bacteraemia and peritonitis models. pLG2 is a 66 kb conjugative pheromone-responsive plasmid of mosaic structure, that transferred in parallel with the PAI, and might have promoted the PAI

horizontal transfer.

These findings demonstrate the horizontal gene transfer of virulence factors and antibiotic resistance gene clusters by a single genetic event, which might be triggered by heavy antibiotic use, common in health care units where enterococci are increasingly prevalent.

Zusammenfassung

Horizontal übertragbare Elemente von Enterococci

Enterokokken (vor allem *Enterococcus faecalis* und *E. faecium*) sind die dritthäufigste Ursache von Krankenhaus Infektionen und erlangen durch ihre schnelle Anpassung an die klinische Umgebung immer größere Bedeutung. Innerhalb der Spezies *E. faecium* und *E. faecalis* existieren Krankenhaus-assoziierte klonale Komplexe, die eine Tendenz zur Ausbreitung, den erhöhten Erwerb an Resistenzmechanismen und eine erhöhte Pathogenitätsausprägung zeigen. Die hohen Rekombinationsraten von Enterokokken sind mit mobilen genetischen Elementen assoziiert, die für Antibiotikaresistenzgene und Pathogenitätsfaktoren kodieren.

Diese Arbeit präsentiert die Ergebnisse der Analyse von zwei Virulenz-assoziierten mobilisierbaren Elementen von *Enterococcus*: das putative Hyaluronidase-kodierende Gen von *E. faecium* (hyl_{Efm}) und die *E. faecalis* Pathogenitätsinsel (PAI), welche das Oberflächenprotein *esp* trägt. Das hyl_{Efm} Gen, welches innerhalb einer putativen genomischen Insel (GI) kodiert ist, kommt in den polyklonalen Krankenhaus-assoziierten Subpopulationen vor. Es wurde festgestellt dass, bei der Mehrheit von 39 hyl_{Efm} positiven Stämmen, die vollständige hyl_{Efm} GI (17824 bp) auf großen Plasmiden (150 bis 350 kb) vorliegt (n = 37). hyl_{Efm}- Plasmide aus *E. faecium* wurden horizontal in verschiedene *E. faecium* Rezipientenstämme aber nicht in *E. faecalis* übertragen. Die Sequenzierung des hyl_{Efm}-Plasmides PLG1 identifizierte putative Gene für die Plasmidreplikation, -Konjugation und -Stabilität, sowie weitere putative Pathogenitätsfaktoren (zB. *pilA*) und Antibiotikaresistenz-Gene (zB. *vanA*, *erm*(B)). Obwohl die *E. faecalis* PAI (153 kb) mobil zu sein scheint, wurden bisher nur interne Fragmente der PAI oder größere chromosomale Regionen, welche die PAI enthalten, mobilisiert. Diese Arbeit zeigt erstmals die horizontale Übertragung des gesamten PAI Elements aus dem Chromosom von *E. faecalis* UW3114 (ca. 200 kb) unter Erythromycin-Selektionsdruck (im Plasmid PLG2). Es konnte die Excision, Zirkularisierung, horizontale Übertragung und Orts-spezifische Reintegration in das Chromosom von *E. faecalis* (intergenisch) und *E. faecium* (tRNALys) gezeigt werden. Mehrere phänotypische Merkmale begleiten den Erwerb der PAI in *E. faecalis*: Expression von Esp auf der Zelloberfläche, verstärkte Bildung von Biofilmen und erhöhte zytotoxische Aktivität. Es wurden jedoch keine Unterschiede in der Patho-

genitätsausprägung im Bakteriämie- und Peritonitis-Mausmodell beobachtet. PLG2 ist ein 66 kb großes, konjugatives Pheromon-Plasmid mit Mosaik-Struktur, das parallel mit der PAI übertragen wurde und könnte den horizontalen Transfer der PAI gefördert haben.

Diese Befunde zeigen den horizontalen Gentransfer von Virulenzfaktoren und Antibiotikaresistenzgen-Clustern in einem einzigen genetischen Ereignis. Solche Ereignisse können besonders in klinischen Einrichtungen, wo Enterokokken in zunehmendem Maße verbreitet sind, durch den erhöhten Einsatz von Antibiotika ausgelöst werden.

1. Introduction

1.1 Enterococci

Enterococci are ubiquitous Gram-positive cocci (Figure 1.1), living in the gastrointestinal tract of humans and animals and are encountered in nearly everything humans come to contact with. Enterococci were formerly classified as group D streptococci until 1984 when genetic evidence was provided that gave them the genus status. They are facultative anaerobes, well adapted to survive in many complex niches and capable of resisting a variety of environmental stresses including heat, acid, oxidation, hyperosmolarity and UV radiation. Enterococci can also grow in broth containing 6.5% NaCl and 40% bile salts, can tolerate ethanol, detergents and prolonged desiccation [reviewed in 34, 115]. In healthy humans, enterococci are constituents of the colon flora at relatively low levels, but it appears that they are the most abundant group of bacteria in the small intestine [54, 160]. However the role that enterococci play in the healthy human gastrointestinal tract is unclear. Currently 40 species in the genus enterococcus have been described (`http://www.bacterio.cict.fr/e/enterococcus.html`). *Enterococcus faecalis* and *E. faecium* are the species most commonly associated with colonization of the human gastrointestinal tract and also cause the vast majority of enterococcal infections.

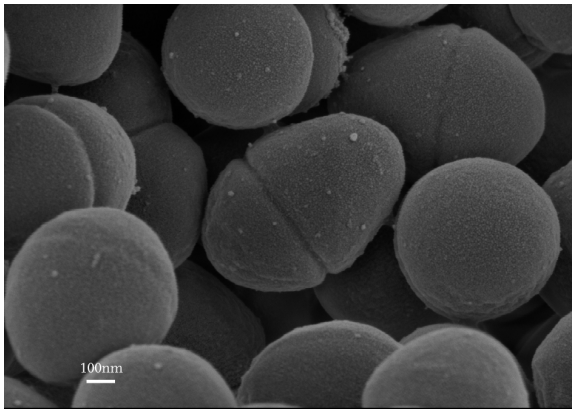


Figure 1.1: Scanning Electron microscopy of *E. faecalis* strain OG1RF (Image: Dr. Norbert Bannert, Robert Koch Institute, Berlin)

1.1.1 Hospital associated enterococci

Although initially regarded as harmless commensals, nowadays infections caused by members of the genus Enterococcus include urinary tract infections (UTI), bacteraemia, intra-abdominal and wound infections and endocarditis, often as part of mixed species infections in immunocompromised patients. Currently, enterococci rank as the third leading cause of nosocomial infections [123]. While *E. faecium* constitutes the majority of vancomycin- and ampicillin-resistant isolates of enterococci, *E. faecalis* accounts for the majority (60-80%) of nosocomial enterococcal infections [reviewed in 115]. *E. faecalis* and *E. faecium* are genetically not much related to each other, as shown by phylogenetic clustering on the basis of 16S rRNA sequences or the sequences of the housekeeping gene encoding the α-subunit of ATP synthase, *atpA* [108]. There are also a number of physiological differences between *E. faecalis* and *E. faecium* such as the ability of *E. faecalis* to produce acid from glycerol, to ferment pyruvate and the presence of cytochrome-like respiratory system and a catalase which are absent from *E. faecium* [67].

1.1.2 *E. faecium*

Since the late 1980s a rapid increase in nosocomial *E. faecium* infections has been observed [172]. The ecological switch among hospital-derived enterococci in favour of *E. faecium* requires special attention because resistance against important antibiotics such as ampicillin and vancomycin emerged predominantly in *E. faecium* and less in *E. faecalis*. Early epidemiological investigations of enterococcal infections were based on phenotypic characteristics. The introduction of various molecular techniques has substantially improved the ability to discriminate enterococcal isolates and has provided critical insights into the epidemiology of enterococcal infections and the population biology at the species level. It has been demonstrated that hospital-associated strains are exogenously acquired and spread by direct and indirect contact among patients and intra hospital transmission and inter-hospital spread have been documented [34]. Macrorestriction analysis using Pulse Field Gel Electrophoresis (PFGE) is a highly discriminative typing method [171, 26, 191]. PFGE can resolve the identity between strains circulating within a confined area or from a localized outbreak. However the high genome plasticity of enterococci, leads to extreme genome variations making PFGE less suitable for long-term epidemiological considerations. Other typing methods as Amplified Fragment Length Polymorphism (AFLP), Multiple Loci Variable number of tandem repeats (VNTR) Analysis (MLVA) and especially Multilocus Sequence Typing (MLST) were established to circunvent limitations of PFGE and allow populational investigations. MLST analysis initially suggested that the majority of infectious isolates of *E. faecium* grouped in a distinct cluster named clonal complex 17 (CC17) [194], however it has been dis-

puted that the e-Burst algorithm initially used to describe the population structure of *E. faecium* was not appropriate as its evolution is driven by high recombination and low mutation rates [175]. Recent analysis of the MLST database for *E. faecium* using more appropriate algorithms has shown that the sequence types constituting CC17 have evolved independently from different ancestral clones and have formed a sub population of hospital associated clones [195]. Hospital- derived strains form a polyclonal subpopulation of *E. faecium* which is linked to vancomycin, high-level ampicillin and high level ciprofloxacin resistance [81, 189]. They are also enriched in virulence associated factors including the Enterococcal surface protein *esp* gene [193, 198, 7], the *E. faecium* hyaluronidase hyl_{Efm} [120], pili [59, 60] and a recently identified gene cluster that is highly specific for hospital-derived *E. faecium* [57].

1.1.3 *E. faecalis*

E. faecalis causes the majority of human enterococcal infections and is by far more pathogenic than *E. faecium*. Vancomycin resistance, seen predominantly in *E. faecium* is an uncommon property of *E. faecalis*. Different MLST schemes have been used to study the epidemiology and population structure of *E. faecalis*. An MLST scheme using seven house keeping genes showed that, in contrast to *E. faecium*, *E. faecalis* obtained from different epidemiological sources (hospitalized patients, community and animals) frequently share identical MLST- sequence types (STs) and group together in common complexes. Despite this alleged random dispersion of human clinical, surveillance and animal isolates, two of the major complexes (CC2 and CC9) contain almost exclusively hospital-derived isolates, suggesting that they are associated with the hospital environment [128].

1.2 Antibiotic resistance in enterococci

Enterococci attract increassing attention because of their increasing role in nosocomial infections and because of their remarkable and increasing resistance to antimicrobial agents. Antimicrobial resistance can be divided into that which is an inherent or intrinsic property and that which is acquired [100].

1.2.1 Intrinsic resistance

Unlike acquired resistance, intrinsic resistance is based in chromosomal genes and is typically non-transferable. Enterococci are inherently more resistant to antimicrobial drugs than other clinically important Gram-positive bacteria [160]. Table 1.1 presents the intrinsic antibacterial drug resistance mechanisms in enterococci. A characteristic feature of enterococci is the resistance to β-lactam antibiotics (particularly cephalosporins and penicillinase-stable penicillins) due to low affinity of the

Drug	Mechanism of resistance
β-lactams	Overproduction of low-affinity PBPs and/or decreased affinity for binding β-lactams
Aminoglycosides	Impermeable cell wall, low uptake and lack of an electron transport chain.
Clindamycin and lincomycin	Low uptake and permeability
Fluoroquinolones	Low permeability, reduced uptake and anaerobic environment.
Trimethoprim-sulfamethoxazole	Resistance *in vivo* due to use of exogenous folates
Glycopeptides	D-Ala-D-Ser ligase (VanC)

Table 1.1: Intrinsic antibacterial drug resistance in enterococci. PBP, penicillin binding protein. [Table from 160]

penicillin-binding proteins [160]. *E. faecium* are less susceptible to β-lactam antibiotics than *E. faecalis* because the penicillin-binding proteins of *E. faecium* have markedly lower affinities for the antibiotics [196]. The first reports of strains highly resistant to penicillin began to appear in the 1980s [12, 134]. Resistance to semisynthetic penicillinase-resistant penicillins is more pronounced. In addition to the high minimmum inhibitory concentrations (MIC), enterococci are "tolerant" to all β-lactams, that is, they are not killed by concentrations higher than the MIC [100].

Another characteristic feature of enterococci is their resistance to the clinically achievable concentrations of the lincosamide antibiotics: clindamycin and lincomycin.

Low level resistance to aminoglycosides is also an inherent property of enterococci and is due to low uptake of these agents. In the presence of cell wall inhibitors such as a β-lactam or a glycopeptide, uptake of the aminoglycoside is markedly enhanced. This results in enhanced killing, the well known synergistic effect [100]. Enterococci intrinsically resistant to vancomycin (IVRE) are infrequently recovered (e.g. 1-2% of enterococcal isolates) and are regarded as not clinically significant. Low level intrinsic resistance has been shown to be associated with serine-containing peptidoglycan in *E. gallinarum* (vanC phenotype). The replacement at the C-terminus of the peptidoglycan precursors D-Ala-D-Ala by D-Ala-D-Ser (produced by the ligases Ddl and VanC1 respectively) results in lower affinity for vancomycin binding. [160]. The vanC cluster consists of five genes, is chromosomally encoded and nontransferable and has been detected on strains of *E. gallinarum*.

1.2.2 Acquired resistance

Enterococci often acquire antibiotic resistance through exchange of resistance-encoding genes carried on conjugative transposons or broad-host-range plasmids. Multi drug resistant (MDR) enterococci have emerged rapidly. The first high level gentamicin resistance occurred in 1979 [65, 201], simultaneously, sporadic outbreaks of nosoco-

mial *E. faecalis* and *E. faecium* infection appeared with penicillin resistance due to β-lactamase production [102] and finally MDR enterococci resistant to vancomycin were reported in Europe [82, 178] and the United States [130]. The emergence of high level ampicillin and vancomycin resistant *E. faecium* as a cause of nosocomial infections has serious therapeutic consequences. Table 1.2 shows the most important known mechanisms of acquired antibiotic resistance in enterococci. Enterococci are intrinsically resistant to β-lactams, but high level resistance is due to overproduction of a PBP with a natural low affinity for penicillins or to mutations that make the PBP even less suceptible to penicillins inhibition. *E. faecalis* strains that produce β-lactamase are fortunately still rare [101]

1.3 Pathogenicity of enterococci

Since the first identification of a vancomycin-resistant enterococci (VRE) in 1986 [82, 178] and with the emergence of multiple drug resistant strains, the question of enterococcal virulence has attracted increased public and clinical interest. Nowadays they represent an increasing problem due to the evident hospital association of certain clones and the therapeutic difficulties linked to the vast array of antibiotic resistance traits that they possess. Initially it was believed that the enterococci native to the patients' intestinal flora were the cause of nosocomial infections. However it has become clear that for multiple antibiotic resistant infections, the situation is more complex. Hospital epidemics are caused by certain circulating clones, in contrast, enterococci from the flora of healthy patients are rarely clonal and rarely antibiotic resistant. Moreover it is known that, within hours to days of admission, the gastrointestinal tracts of patients become colonized with exogenously acquired MDR strains [95, 43, 195]. Therefore, a prerequisite for multiple antibiotic resistant enterococcal infections appears to be the asymptomatic colonization of the GI tract of patients following hospital admission [115]. The search for factors that subvert the commensal relationship has lead to the finding and characterization of pathogenicity factors as traits that a) are not required for commensal existence as evidenced by their absence from most of commensal isolates, b) are part of the accesory genome or c) contribute to the severity of enterococcal infection in one or more models of enterococcal infection. Further additional factors have been described that contribute to the fitness of enterococci at the site of infection and therefore constitute legitimate therapeutic targets, but the role in the commensal existence of enterococci is less clear because of widespread and distribution within the genus or species, including commensal strains [41]. Table 1.3 resumes the enterococcal pathogenicity factors known until now.

Antibiotic resistance	Mechanism
HLR to aminoglycosides [a]	Enzymatic (production of aminoglycoside modifying enzymes)
Gentamicin	Phosphotransferases (APH)
Kanamycin	Acetyltransferases (AAC)
Streptomycin	Nucleotidyltransferases (ANT)
	Alteration of the target (leading to decreased ribosomal binding)
Resistance to glycopeptides[b]	Alteration of the target (modification of the peptidoglycan biosynthetic pathway)
Vancomycin, Teicoplanin	
Van A, Van B	
Van C (VanC1, VanC2, VanC3)	
Van D, Van E, Van G	
Resistance to β-lactams[c]	Alteration of the target (altered penicillin binding proteins (PBP), in particular PBP5)
Penicillin	
Ampicillin	Enzymatic (production of β-lactamase)
Resistance to quinolones	Target alteration (changes to the subunit A of DNA gyrase (GyrA) and topoisomerase Ivsubunit (ParC))
Resistance to chloramphenicol	Enzymatic (production of chloramphenicol acetyl transferase)
	Active efflux
Resistance to the MLS group	
Macrolides (erythromycin)	Enzymatic (production of methylating enzymes)
	Efflux protein pump (ATP-binding transporter protein)
Lincosamides (clindamycin HLR)	Nucleotidyltransferase (adenylation of an hyldroxyl group in LIN and CLI)
Streptogramin B	Acetyltransferases
Tetracyclin	
tet(K), tet(L)	Active efflux
tet(M), tet(N) tet(O), tet(S)	Ribosomal protection (modification of ribosomal conformation)
Oxazolidinone	Alteration of the target (decreased
Linezolid	ribosomal binding through 23s rRNA mutations)
Everninomicins	Alteration of the target (rRNA methyltransferase-mediated 23s rRNA methylation)

Table 1.2: Major patterns and mechanisms of acquired resistance to antimicrobial agents in enterococci. [a]HLR, high level resistance; found in increasing frequencies in *E. faecalis* and *E. faecium*. [b] VanA and VanB phenotypes are usually found in *E. faecalis* and *E. faecium*; VanD in *E. faecium* and VanE in *E. faecalis* BM4405; VanC1 is associated with *E. gallinarum*, VanC2 and VanC3 with *E. caseliflavus*. [c] Found in *E. faecalis* and *E. faecium*. [Table modified from 34]

1.3.1 Enterococcal surface protein, Esp

The enterococcal surface protein Esp is a cell surface-localized, 1,873 amino acid-long protein of approximately 200 kDa that is coded by the *esp* gene and is expressed on the surface of *E. faecium* (esp_{Efm} gene) and *E. faecalis* [80, 33, 144]. While *esp* is widely distributed among *E. faecalis* strains, it appears to be associated to epidemic (vacomycin-resistant) *E. faecium* strains [80, 193]. Esp proteins contain an N-terminal signal sequence followed by 3 to 11 very highly conserved tandem repeats 82 amino acids in length. It possesses a transport signal sequence and a cell wall anchor motif [144]. In *E. faecalis*, experimental evidence has been provided about the role of Esp as an adhesin in colonization of the urinary tract of mice. [142]. It has been demonstrated that although *esp* is capable of increasing biofilm forming capacity in *E. faecium* and *E. faecalis* [161] [170] [180] [56], it is neither necessary nor sufficient for biofilm formation [77] [124]. The *esp* gene is located on two different pathogenicity islands (PAIs) in both *E. faecalis* and *E. faecium*.

1.3.2 Putative hyaluronidase hyl_{Efm}

Recently an ORF with significant deduced amino acid identity to hyaluronidase genes has been found in the multiresistant strain *E. faecium* C68 (DO) and was designated hyl_{Efm} [120]. Hyaluronidases are putative virulence factors that play a role in adhesion or colonization [70], tissue degradation and spread [114]. So far no biochemical evidence for the actual enzymatic activity of hyl_{Efm} has been reported. hyl_{Efm} might constitute a family 84 glycoside hydrolase with β-N-acetylglucosaminidase activity as indicated by sequence similarities to such proteins [39]. Since then several molecular and epidemiological studies have revealed the almost exclusive presence of hyl_{Efm} among clinical strains as well as an increase in the number of CC17-*E. faecium* carrying hyl_{Efm} in hospitals of different countries [120, 173, 195, 27, 39]. While several studies demonstrate the epidemiological link between hyl_{Efm} and infectious isolates, the demonstration of hyl_{Efm}-associated pathogenicity remains unresolved. A hyl_{Efm} deletion mutant of *E. faecium* demonstrated attenuation compared to the wild type in a mouse peritonitis model, however complementation studies of the hyl_{Efm} mutation were missing. In the same study pre-immunised mice with anti-hyl_{Efm} immune rabbit antisera were challenged with a wild type *E. faecium* hyl_{Efm} positive strain, demonstrating protection of infection [4]. Recent studies have demonstrated that hyl_{Efm} is plasmid-located and that the horizontal acquisition of the hyl_{Efm}-containing plasmid confers strains an increased virulence in a mouse peritonitis model; this evidenced a role in pathogenicity for hyl_{Efm} plasmids but it could not be exclusively linked to hyl_{Efm} presence [3, 121].

	Pathophysiology/Virulence	Epidemiology	Reference
***E. faecium* (Efm)**			
Enterococcal surface protein, Esp	Biofilm formation, Pathogenesis, Antigenic	Specifically linked to HA Efm	56, 83
Collagen binding adhesin of Efm, Acm	Binding to collagen. Pathogenesis, Antigenic	Widespread among Efm	104, 106, 105
Pili PilA PilB	Not known	Widespread among Efm	59, 61
Second collagen adhesin, Scm	Binding to collagen	Widespread among Efm	148
collagen binding adhesin, EcbA	Binding to collagen and fibrinogen	Specifically linked to HA Efm	60, 61
hyaluronidase (hyl_{Efm})	Putative ECM degradation	Specifically linked to hospital associated clones (CC17)	120, 39, 3, 121
***E. faecalis* (Efs)**			
Agregation substance, AS: Asa1, Asp1, Asc10	Bacterial aggregation, conjugation. ECM binding. Internalization, pathogenesis.	Widespread among Efs	112, 184, 135, 188, 76, 158, 126, 16, 138, 182, 119, 63
Esp	Biofilm formation. Pathogenesis	Widespread among Efs	142, 161, 162, 170
Gelatinase/quorum sensing locus *fsr*	Biofilm formation Pathogenesis.	Widespread among Efs	10, 117, 152, 147, 103
Pili *ebp* locus	Biofilm formation. Pathogenesis, Antigenic	Widespread among Efs	107, 163, 150
bee locus	Biofilm formation	Among 5% of Efs	21
Adhesion to collagen, Ace	Binding to collagen, laminin and dentin	Widespread among Efs	122, 75, 104, 151
capsular polysaccharide, Cps	Antiphagocytic. Immunologic evasion	Efs serotype C and D strains	66, 50, 168, 169
enterococcal polysaccharide antigen, epa	Biofilm formation, translocation, Pathogenesis	Present in Efs cell wall	199, 164, 200, 149
Lipoteichoic acid, LTA (enterococcal group antigen)	Conjugation (binding), Inmunogenic.	Present in enterococcal cell wall	165, 138
Wall teichoic acid WTA	Not known	Present in enterococcal cell wall	109
Glycolipids	Biofilm formation. Colonic cells adherence. Pathogenesis	Present in enterococcal cell membrane	137, 166
Cytolysin (hemolysin/ bacteriocin)	Lysis of eukaryotic and Gram positive cells	Widespread among Efs	16, 23, 24
Reactive oxigen species	Extracellular superoxide, hydrogen peroxide	Widespread among Efs. Less frequently also found in Efm	68, 96, 69

Table 1.3: Putative virulence factors in *E. faecalis* and *E. faecium*. UTI: urinary tract infection. HA: hospital-associated. PMNs: polymorphonuclear leukocytes. ECM: extracellular matrix. [Table modified and completed from reference 136].

1.4 Horizontal gene transfer and mobile genetic elements

The horizontal gene transfer between microorganisms has a great impact on the evolution of bacterial pathogens. The genome of bacterial pathogens is composed of a conserved "core" genome, which contains the genetic information that is required for essential cellular functions and an "accesory" gene pool, which encodes additional traits that might be beneficial under certain circumstances. The accesory gene pool represents mainly mobile elements including bacteriophages, plasmids, genomic islands (GIs), insertion sequence (IS) elements and various types of transposons [31, 25, 55]. Virulence genes are frequently located on mobile or formerly mobile, genetic elements including PAIs. The medical importance of the enterococci is closely related to their propensity to participate in the horizontal transfer of determinants for antibiotic resistance and virulence. Transfer involves primarily conjugation systems which are abundant and involve plasmids and conjugative transposons. There are no reports of natural transformation or transduction in these organisms.

1.4.1 Enterococcal plasmids

Clinical isolates of enterococci carry up to five or six co-resident plasmids although in most cases little is known of their classification genetic content. Determinants for antibiotic resistance, haemolysins, bacteriocins and resistance to ultraviolet light are frequently plasmid associated and many such plasmids encode the ability to transfer or may be efficiently mobilized by a co-resident conjugative element [20] Three classes of plasmids are known to be capable of replication in the enterococci: the pheromone responding plasmids, the rolling circle (RCR) plasmids and the Inc18 plasmids. The RCR and Inc18 plasmids are capable of replication in a broad range of Gram-positive bacteria and some RCR are also capable of replication in Gram-negative species. The pheromone responding plasmids appear to be restricted to the enterococci [186]. Large plasmids in enterococci were not described until recently [186], however according to recent reports it seems like they are also widely disseminated [39, 179]. RCR plasmids are a rather large group of small, high-copy plasmids that replicate via a rolling circle mechanism. The RCR process of unidirectional nucleic acid replication can rapidly synthesize multiple copies of circular molecules of DNA or RNA. Originally identified in *Staphylococcus aureus*, RCR plasmids are found in virtually all Gram-positive bacterial species [186]. A number of cloning, expression, transposon delivery and replicon-probe vectors derived from RCR plasmids have proven useful in the study of enterococci. The Inc18 plasmids are a family of plasmids between 25 and 30 kb in size, widely distributed among the low G+C Gram-positive bacteria. Many are self conjugative and are maintained at a relatively

low copy number (< 5 copies/cell) The pheromone responding plasmids are characterized by the ability of their hosts to form mating aggregates when mixed with plasmid free cells (recipients) in broth. A peptide pheromone is secreted by plasmid free recipient cells. Once a given pheromone responding- plasmid is acquired, production of the related pheromone is shut down, however unrelated pheromones (at least six) specific for a different family of donor plasmids, continue to be secreted. The formation of mating aggregates relates to the induction of the protein "aggregation substance" (AS) (see also 1.3), which appears extensively over the donor surface and binds to "enterococcal binding substance" (EBS). The prototype of pheromone responding plasmids are pAD1 (60 kb), pCF10 (65 kb), pPD1 (56 kb) and pAM373 (36 kb). All reported pheromone responsive plasmids are from *E. faecalis*, with the exception of pHKK100 which is from *E. faecium* [reviewed in 20].

1.4.2 The *E. faecium* hyl_{Efm} genomic island

As previously mentioned, hyl_{Efm} is a putative virulence gene which predominates in *E. faecium* of clinical origin. hyl_{Efm} was intially described as part of a putative pathogenicity island, ca. 20- kb, that is flanked by inverted copies of putative insertion sequence IS*1476*. Within the supposed GI, upstream of hyl_{Efm} exist similarities to 2 component regulator systems and a β-galactosidase gene. Downstream of hyl_{Efm} lies an ORF with no significant homologies and an ORF encoding a putative protein with homology to previously described guanine monophosphate (GMP) synthases [120].

Recently, it has been described that hyl_{Efm} is contained within a 220 kb transferable plasmid in *E. faecium* strain C68, that can increase the virulence of strains. The plasmid location of hyl_{Efm} was demonstrated in other strains and a physical link to vancomycin resistance genes, high level resistance (HLR) to amynoglycosides (gentamycin and streptomycin) and erythromycin was shown [3]. Analysis of 51 hyl_{Efm} positive strains showed that hyl_{Efm} is located in megaplasmids ranging from 170 kb to 375 kb which do not contain replication proteins of the most known Gram-positive plasmids. These plasmids showed frequent co-localization of *erm*(B) but not of *vanA* genes [39]. Horizontal transmission of hyl_{Efm} plasmids has been previously described both when hyl_{Efm} and *van* are genetically linked [3, 121] and when they are not [39].

1.4.3 Pathogenicity islands transfer

PAIs are large genomic regions (10-200 kb) that are present in pathogenic variants but less frequently in closely related non-pathogenic bacteria. PAIs carry one or more virulence-associated genes, have a G+C content that differs from that of the rest of the chromosome and are frequently integrated into tRNA genes [reviewed

in 31]. PAIs are normally flanked by short direct repeats (DR) resembling attachment sites for phage integrases (attL and attR) and carry phage related integrase genes. This suggests that PAIs derive from integrating plasmids or phages and that their mechanism of integration and excision corresponds to that of integrative and conjugative elements (ICEs) [47].

For ICEs mobilization, the excisionase- mediated homologous recombination between *attL* and *attR* produces excision and formation of circular intermediates (carrying *attB*), while a recombinase (integrase) catalyzes integration by recombination between *attB* and a chromosomal target sequence *attP* generating the junction sequences *attL* and *attR* [11]. The majority of PAIs described to date have degenerate integrase genes or recombination sites but direct proof of integration and excision is lacking [177]. Several PAIs have been reported to be capable of spontaneous excision from the chromosome and formation of circular intermediates, which are thought to be prerequisite for the horizontal transfer. These events have been described for the *Vibrio cholerae* PAIs (VPI) VPI-1, VSP-I and VSP-II [99, 118], the *Salmonella* GI 1 (SGI 1), [32], the *Yersinia pseudotuberculosis* high pathogenicity island (HPI) [131], the *Shigella flexneri* PI (*she* PAI), the locus of enterocyte effacement (LEE) of a rabbit-specific enteropathogenic *Escherichia coli* strain [97] and the six PAIs of *E. coli* isolate 536 (PAII536 to IV536) [93, 64].

Horizontal transfer of PAIs into recipient strains facilitated by phages has been described in VPI-1 [113] and SaPI-1 [129]. Similarly, plasmids harbouring regions of three SaPIs, of the *E. coli* O26 locus of enterocyte effacement (LEE O26) and of *she*PAI demonstrated PAI integration into the chromosome of recipient strains [177, 97, 131], while SGI1 and the *E. coli* HPI have been shown to transfer with the help of plasmids [32, 139]. So far, only the *Y. tuberculosis* HPI has been observed to transfer spontaneously in a liquid medium without the help of a phages or plasmids [85]. Previous attempts to transfer precisely the *E. faecalis* PAI have failed; only parts of the PAI or larger chromosomal elements containing the PAI have been observed to transfer [111, 22, 89].

Some environmental stresses that stimulate horizontal transfer of island-like elements have been identified. Mobilization of temperate phages and ICEs involves the SOS response to mobilize themselves from the chromosome, induced by e.g. UV irradiation or bactericidal antibiotics [9, 53, 92, 176]. The SOS system represents a global response to DNA damage that upregulates genes involved in DNA repair recombination and mutagenesis [29, 28]. The SOS response has been shown to induce the lateral transfer of antibiotic resistance encoded by the Vibrio cholerae integrating conjugative element SXT (quinolone induced) [9] and of prophage-encoded Shiga toxin in *E. coli* (fluoroquinolone induced) [203]. At subinhibitory concentrations, quinolones have been shown to induce partial or total loss of PAIs *in vitro* in uropathogenic *E. coli* by SOS-dependent or -independent pathways, respectively [156]. Fluoroquinolones (ciprofloxacine) are fully effective for the mobilization of

the staphylococcal pathogenicity islands SaPIbov1 and SaPI1 and, by implication, of all other SOS induced SaPIs [176]. β-lactam antibiotics induce an SOS response in *S. aureus* that results in replication and high-frequency transfer of the SaPIbov1 and SaPI1 [88].

The *E. faecalis* pathogenicity island

Genomic analysis of *E. faecalis* strain MMH594, which caused an outbreak in the mid 1980s and strain V583, the first vancomycin resistant isolate in the United States, revealed the presence of a PAI of variable content. The genetic element in MMH594 is 153,571 bp large, codes for 129 open reading frames (ORFs) and possesses all the hallmarks of a PAI. These include flanking DR, variant G+C content (32.3% compared to the 37.38% of the whole genome) and the presence of putative genes encoding an integrase, an excisionase, transposases, transcriptional regulators and proteins with potential roles in virulence or adaptation and survival. The *E. faecalis* PAI is inserted in a non-coding region flanked by an ORF coding for a hypothetical protein of unknown function and a putative oxidoreductase related to the aldo-keto reductase family, differing from most of bacterial PAIs that insert within transfer RNA [141]. The *E. faecalis* PAI encodes variable traits of *E. faecalis* associated with virulence, including cytolysin, aggregation substance and Esp. Additionally within the element are genes that seem to code for a DNA-damage inducible protein, an AraC-like transcriptional regulator, a conjugated bile acid hydrolase, components of the phosphotransferase system and a GLS-24 like starvation inducible protein. It appeared as if the 5' portion of the island was derived from integration of a conjugative plasmid into the chromosome, since it bears elements of pheromone responsive plasmids pAM373 and pAD1. This plasmid-like region contains genes encoding an aggregation substance and a TraG-like protein apparently derived from pAM373, the cytolysin operon and a region with 87% identity to an *oriT* derived from pAD1 [141]. The *E. faecalis* PAI is widely distributed among isolates of different origins, clonal types and complexes and its content varies greatly between different isolates [91, 1, 90, 143]. This seems to be an speciality of the *E. faecalis* PAI and contrasts the situation in Gram-negative bacteria where PAIs don't have a modular structure or variable gene content. Mc Bride *et al.* have compared among a collection of strains from different origin, the structural variations of the PAI, using microarray analysis, to the relatedness and chromosomal variations investigated by MLST. The results indicated that the presence of specific PAI regions in different strains does not correlate closely with the predicted relatedness of strains. It is supposed that core elements of the PAI radiated outward through the species and in the process it continues to evolve by modular gain and loss of internal gene clusters, in a more rapid way than the core genome does [90]. The first two genes within the PAI are a putative phage related integrase and an excisionase and a 10 bp duplicated sequence

flanks the PAI integration site [141]. These, together with the presence of putative plasmid-derived transfer functions, suggest that the PAI was integrated and might be excised and transferred as a single entity [22]. Several studies have pursued the horizontal transfer of the *E. faecalis* PAI. Oancea *et al.* showed that the *esp* gene could be transferred among *E. faecalis* (and also among *E. faecium*) when selecting for the transfer of antibiotic resistance determinants. The *E. faecalis esp* transferred from chromosome to chromosome, however whether the whole or further fragments of the *E. faecalis* PAI had also been transferred, could not be investigated. Coburn *et al.* used a *cat* marker to tag the *E. faecalis* PAI and observed that during filter mating experiments, the chloramphenicol resistance was transferred to strain OG1RF. A secondary transfer into strain JH2SS was also achieved. Analysis of the transconjugants demonstrated that a 27,774 bp internal PAI segment was capable of excision and circularization in the donor and was mobilized as a cointegrate with a pTEF1-like plasmid. Very recently Manson *et al.* have reported the horizontal transfer of the *E. faecalis* PAI not exclusively as a self excisable mobilizable element but always accompanied by flanking donor chromosome sequences. The horizontal transfer of other chromosomal regions was also observed, always accompanied by the transfer of a pheromone responsive- plasmid (pTEF2 or pTEF3). Deletion experiments showed that the transfer of regions bearing the PAI did not require the integrase and excisionase genes encoded within the PAI, whereas deletion of plasmid transfer functions, including the cis-acting origin of transfer (*oriT*), abolished movement. These findings evidenced the role of plasmids in the mobilization of large genomic elements and evolution of *E. faecalis* [89].

In *E. faecium* the esp_{Efm} is carried on a large PAI structure (between 64 and 104 kb) with some conserved parts and variable presence of some genetic elements. The C+C content of the PAI is 2.3% lower than that of the entire genome, it is flanked by an imperfect 54-bp DR and is integrated downstream of the *tuf* or the *rpsI* gene. A 10 kb element of the esp_{Efm}PAI is found identical in the 154 kb *E. faecalis* *esp*-carrying PAI, suggesting a recent transfer of genetic material between these two species or acquisition from a third source [80, 179].

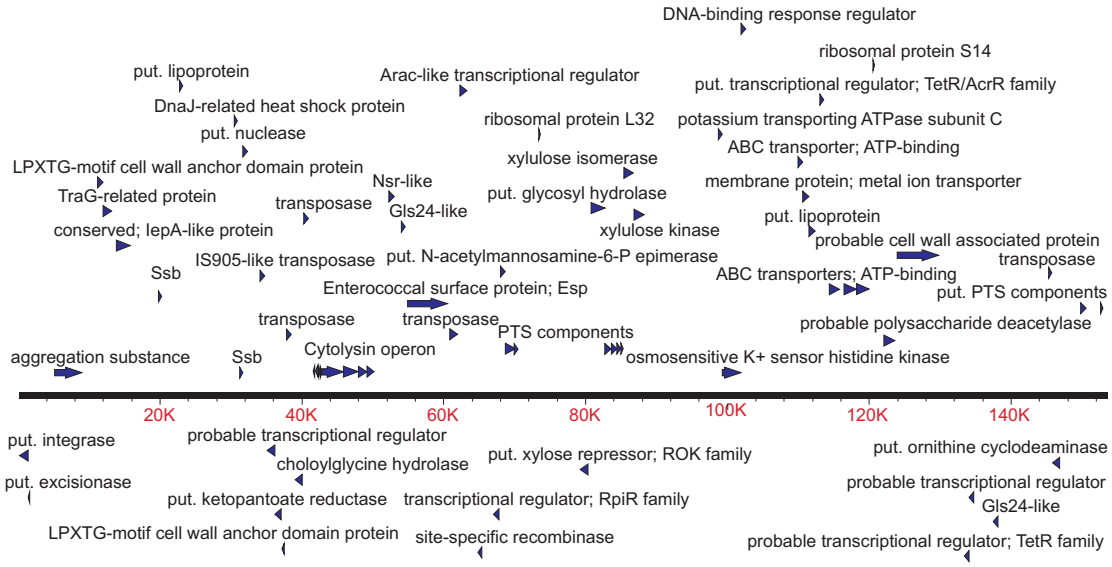


Figure 1.2: The *E. faecalis* PAI structucture of strain MMH594. Figure based on the available sequence ($gb|AF454824.1|$), displaying all putative pathogenicity factors contained. ORFs encoding hypothetical proteins are not shown.

2. Objectives

Horizontal transfer of the accesory genome plays a very important role in the adaptation and increasing relevance of enterococci as nosocomial pathogens. However, the transfer of pathogenicity islands among enterococci has been scarcely investigated and still is not well understood. Similarly, enterococcal megaplasmids bearing putative pathogenicity factors (as hyl_{Efm}) has been just recently reported and its biology, classification and significance remain unknown. The overall aim of this thesis was to study the intra and inter species horizontal transfer of two elements of the accesory genome of enterococci: the *E. faecalis* PAI and the plasmid encoded-hyl_{Efm} putative genomic island.

More specifically, the following objectives were pursued:

- Investigate the horizontal transfer of the pathogenicity island of *E. faecalis* intra and inter species to *E. faecium*, describing events associated with its chromosomal excision, transfer and integration into recipient strains.
- Analyze the genotypic and phenotypic consequences of the aquisition of the *E. faecalis* PAI in *E. faecium* and *E. faecalis*.
- Investigate the structure, distribution and transfer of the hyl_{Efm} putative genomic island among *E. faecium*.
- Resolve the composition of a hyl_{Efm} megaplasmid identifying those elements that could explain its origin and its specific distribution among clinical strains.

3. Materials and methods

3.1 Media and chemicals

The chemicals, culture media and antibiotics used in this work are listed in Tables 3.1, 3.2 and 3.3. The composition of standard solutions employed is also presented.

Chemical	Producer/Manufacturer
I-*Ceu*I	New England Biolabs Inc.
*Sma*I	New England Biolabs Inc.
Agarose Type	Sigma-Aldrich GmbH
Ammonium citrate	Jenapharm Laborchemie
anti-DIG-AP	Roche Diagnostics GmbH
AttoPhos solution	Roche Diagnostics GmbH
Blocking reagent	Boehringer Ingelheim GmbH and Co. KG
Boric acid	Serva Electrophoresis GmbH
Bovine Serum Albumin (BSA)	Sigma-Aldrich GmbH
Brij®58	AppliChem GmbH
Bromophenol blue	Sigma-Aldrich GmbH
CDP-Star	Roche Diagnostics GmbH
Chloroform	Fluka Chemie AG
Chloroform	CARL ROTH GmbH and Co KG
D-glucose	Merck KGaA
DIG antibodies	Roche Diagnostics GmbH
DIG Easy Hyb Granules	Roche Diagnostics GmbH
Distilled water	Sigma-Aldrich GmbH
EDTA (Ethylenediaminetetraacetic acid)	Serva Electrophoresis GmbH
EDTA disodium (Disodium dihydrogen ethylenediaminetetraacetate)	Serva Electrophoresis GmbH
Ethanol (98%)	Merck KGaA
Ethidium Bromide (10 mg/ml)	CARL ROTH GmbH and Co KG
Glacial acetic acid	Fluka Chemie AG
Glutaraldehyde	Sigma-Aldrich GmbH
glycine	CARL ROTH GmbH and C KG
goat anti-rabbit fluorescein isothiocyanate	Sigma-Aldrich GmbH
Gram's Crystal violet solution	Merk KgaA
hyaluronic acid salt from S. equi	Sigma-Aldrich GmbH
hyaluronic acid salt from umbilical cord	MP Biomedicals LLC
hyaluronidase	MP Biomedicals LLC
Hydrochloric acid (37%) HCl	CARL ROTH GmbH and Co KG
LMP agarose	Biometra GmbH

Lysozyme	Sigma-Aldrich GmbH
Magnesium chloride $MgCl_2$	CARL ROTH GmbH and Co KG
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	CARL ROTH GmbH and Co KG
Maleic acid	CARL ROTH GmbH and Co KG
Methylcellulose 2% 25 centipoises	Sigma-Aldrich GmbH
Phenol / chloroform / isoamyl alcohol (25:24:1)	CARL ROTH GmbH and Co KG
Polyclonal anti-Esp rabbit immnune antiserum	Eurogentech S.A.
Polyclonal anti-pilA rabbit immune antiserum	Eurogentech S.A.
Polyethylene glycol 4000 (50%)	Merck KGaA
Potassium acetate CH_3CO_2K	Merck KGaA
Potassium hydroxide KOH	Jenapharm Laborchemie
Protein A-gold (15 nm)	
Proteinase K	Sigma-Aldrich GmbH
RNaseI	Sigma-Aldrich GmbH
SDS (sodium lauryl sulfate)	CARL ROTH GmbH and Co KG
SI-nuclease	(Takara, Bio Inc., Shiga, Japan)
Sodium acetate $C_2H_3NaO_2$	Merck KGaA
Sodium azide NaN_3	VEB Sprengstoffwerk
Sodium chloride NaCl	CARL ROTH GmbH and Co KG
Sodium citrate $NaH_2C_6H_5O_7$	Jenapharm Laborchemie
Sodium deoxycholate	CARL ROTH GmbH and Co KG
Sodium hydroxide NaOH	CARL ROTH GmbH and Co KG
Sodium-Lauroylsarcosine	Sigma-Aldrich GmbH
Tris	CARL ROTH GmbH and Co KG
TrisHCl	Merck KgaA
Triton X-100	Sigma-Aldrich GmbH
Tween 20	CARL ROTH GmbH and Co KG
Uranyl acetate	Sigma-Aldrich GmbH
DNA Molecular Size markers	
GeneRuler 100bp DNA-Ladder Plus	fermentas GmbH
GeneRuler 1kb DNA-Ladder Plus	fermentas GmbH
DIG III DNA-Ladder	Roche Diagnostics GmbH

Table 3.1: Chemicals and materials used in this work

Medium	Producer/Manufacturer
Brain Hearth Infusion (BHI) agar	Difco® labs.
Brain Hearth Infusion (BHI) broth	Difco® labs.
Dubelco's Modified Eagle's Medium (DMEM)	Difco® labs.
Mueller Hinton (MH) Agar with Sheep Blood	Oxoid GmbH
RPMI 1640	Cambrex Bioscience
Todd-Hewitt Broth (THB)	Bacto™
Tryptic Soy Agar (TSA)	Difco® labs.
Tryptic Soy Broth (TSB)	Difco® labs.

Table 3.2: Media used for the culture of bacteria and animal cells

Antibiotic	Soluble in	Concentration	Producer/manufacturer
Ampicillin	H_2O	8mg/L	Sigma-Aldrich Chemie GmbH
Ciprofloxacine	0.1N HCl	1mg/L	Fluka Chemie AG
Erythromycin	Ethanol	5mg/L	Abbott Laboratories
Fusidic acid	H_2O	20mg/L	Sigma-Aldrich Chemie GmbH
Rifampicin	0.1N HCl	30mg/L	Sigma-Aldrich Chemie GmbH
Spectinomycin	H_2O	300mg/L	Sigma-Aldrich Chemie GmbH
Streptomycin	H_2O	500mg/L	Sigma-Aldrich Chemie GmbH
Vancomycin	H_2O	5mg/L	Sigma-Aldrich Chemie GmbH

Table 3.3: Antibiotics, used in this study, their solubility and concentrations used.

Standard Solutions:

Phosphate Buffer Saline (PBS)	
Sodium chloride	137 mM
Potassium chloride	2.7 mM
Sodium phosphate dibasic	10 mM
Potassium phosphate monobasic	2 mM
pH 7.4	
TES buffer	
Tris-HCl pH 8.0	10 mM
Bacto Trypton pH 8.0	1 mM
Saline Solution	
NaCl	85%
H_2O	
Tris-EDTA (TE) buffer	
Tris-HCl	200 mM
EDTA	20 mM
pH 7.5	

3.2 Equipment and materials

Equipment, electric devices and different materials used in this study are listed in Table 3.4.

Device Material	Producer Manufacturer
200-mesh Formvar-carbon coated grids	
aCOLyte Colony Counter	Don Whitley Scientific Ltd.
Autoclave, Biomedis	H+P Labortechnik
Automatic spiral plater WASP2	Don Whitley Scientific Ltd.
Bag sealer	CARL ROTH GmbH and Co KG
Biodocumentation luminiscence detector ChemiDocXR	Bio-Rad Laboratories
Cellulose nitrate filter, Pore size: 0.45 µm, ⊘ 45 mm	CARL ROTH GmbH and Co KG
Centrifuge (Rotor 12159)	Sigma Laborzentrifugen GmbH
Centrifuge tubes (15 ml, 50 ml)	CARL ROTH GmbH and Co KG
Centrifuge, mini spin plus	Eppendorf AG
Chef DR®III System	Bio-Rad Laboratories
Electrophoresis chambers, Sub Cell Mini and Mini Wide	Bio-Rad Laboratories
Electrophoresis Power suply	Bio-Rad Laboratories
ELISA reader, Sunrise	Tecan Group
Eppendorf tubes (0.2 ml, 0.5 ml, 1.5 ml)	Eppendorf AG
FACSCalibur flow cytometer	BD Biosciences
Filter mating aparatus	Sartorius GmbH
Fine Scale, Genius	Sartorius GmbH
Fluorescence reader, Fluorimeter FLA-2000	Fujifilm
Freezer -20℃, LIEBHERRcomfort	Liebherr-holding GmbH
Glycerin culture tubes	MAST Diagnostica GmbH
Hybridization oven and flasks, Techne	Biostep GmbH
Incubator Shaker Sartorius Certomat	NEW BRUNSWICK SCIENTIFIC, Inc
Incubator shaker SM30	Edmund Buhler GmbH
Incubator, HERAcool	Heraeus Instruments
Jeol 1010 transmission electron microscopy	Jeol-Europe Ltd.
Microbiological Safety Cabinet HERAsafe	Thermo Scientific Inc.
Microwave oven, Severin	CARL ROTH GmbH and Co KG
Multicanal-pipette (8 and 12 canals)	Eppendorf AG
Multistep-Pipette	Eppendorf AG
Nylon membranes	Roche Diagnostics GmbH
Petri plates	Sterilin Ltd.
pH-Metter, HANNA instruments	neolab GmbH
Pipette tips (1 µl, 100 µl, 1000 µl)	Eppendorf AG
Pipettes, Reference, various	Eppendorf AG
Pipettes, Research, various	Eppendorf AG
Plataform shaker, Heidolph Polymax 1040	neolab GmbH
Polystyrene Cell culture microplates 24-well Greiner bio-one AG	
Polystyrene microtiter plates flat bottomed 96-well	Greiner bio-one AG
Polystyrene microtiter plates flat bottomed 96-well	Corning Life Sciences
Precision Scale, Kern 440-43N	Sartorius GmbH

Refrigerator + 4℃, Bosch	Robert Bosch GmbH
Spectrophotometer, NovaspecII	Pharmacia Biotech Inc.
Spectrophotometer, SmartSpect 3000	Bio-Rad Laboratories
Sryinges 5 ml, 10 ml	CARL ROTH GmbH and Co KG
Thermocycler Gene Amp 9700	Applied Biosystems
Thermocycler TGradient	Biometra GmbH
Thermomixer, Comfort	Eppendorf AG
Thermopapier, Mitsubishi	MS Laborgeräte GmbH
Thermo-Printer, Mitsubishi	Bio-Rad Laboratories
Ultraviolet irraadiator, Bio-Link Cross linker BLX 365 nm	Vilber Lourmat
Vacuum Blotter TDNA	Apligene-Oncor
Vacuum centrifuge, concentrator 5301	Eppendorf AG
Variable Speed Pump	Bio-Rad Laboratories
Vortex Genie 2	Scientific Industries
Water bath	GFL GmbH
Water purification system	SG Reinstwasser GmbH
Wattmann Paper 3MM	Whatman Ltd.

Table 3.4: Instruments, devices and materials used in this study

3.3 Software and internet resources

Different software programs and other internet resources used in this study are presented in Tables 3.5 and 3.6.

3.4 Kits

Different kits used for various molecular approaches in this work are listed in table 3.7.

3.5 Primers

All primers used in this work were purchased from Invitrogen, Life Technologies and are listed in Tables A. 1, A. 2, A. 4 and A. 3.

3.6 Filter mating assays

Filter conjugation allowing cell-to-cell contact was used for transferring the *esp*-pathogenicity island (PAI) of *Enterococcus faecalis* and the putative hyl_{Efm} PAI of *E. faecium* by a method previously described [192]. Overnight cultures of the recipient and donor strains at 37℃ in Brain Hearth Infusion (BHI) broth were tenfold diluted in 5ml /BHI broth and growth until exponential phase (3-4h). 1 ml of the donor and 1 ml of the recipient culture were mixed in one tube and applied

Name	Aplication	Developer
BioNumerics 6.0	PFGE analysis, fragment size calculation, phylogenetic analysis	Applied Maths
Quantity One 4.6.6	Documentation of gel images and Southern hybr. Membranes	BioRad Laboratories
Dsgene	Analysis of DNA sequences, graphics	Accelrys, Inc.
AIDA Image Analyzer3.52	Evaluation of Florometric analysis	Raytest
Lasergene8 Seqman	Evaluation of DNA sequences	DNAStar
Lasergene8 MegAlign	DNA alignements	DNAStar
Lasergene8 Seqbuilder	Analysis of DNA sequences, graphics	DNAStar
Lasergene8 PrimerSelect	confirmation of selected primers	DNAStar
Kodon 3.61	DNA sequence alignements, comparissons, CDS prediction, Annotation, graphics	Applied Maths
PRISM4	Statistical analysis	GraphPad Software
Microsoft office	Excel, Word, PowerPoint.	Microsoft
TEXmaker 1.7	Open Source LaTeX word processor	Pascal Brachet
Artemis12	DNA sequence edition	Sanger Institut
Sequin9.00	DNA sequence anotation, submission	NCBI
CCL Main Workbench	phylogenetic analysis	CCL Bio
MEGA	phylogenetic analysis	[159]

Table 3.5: Software programs used in this study.

Name	Aplication
	Internet adress
Primer3	Primer design http://frodo.wi.mit.edu/primer3/input.htm
PrimerBlast	Primer design http://www.ncbi.nlm.nih.gov/tools/ primer-blast/index.cgi?LINK_LOC=BlastHome
NEB Cutter V2.0	In silico restriction of DNA sequences http://tools.neb.com/NEBcutter2/index.php
Annotation2Excel	Conversion of feature tables in Excel tables http://nbc3.biologie.uni-kl.de/
BLAST	Blast search of DNA or protein sequences among all available DNA, protein and translated protein sequences http://blast.ncbi.nlm.nih.gov/Blast.cgi? CMD=Web&PAGE_TYPE=BlastHome [203]
NCBI database	Sequences Database http://www.ncbi.nlm.nih.gov/guide/
Pubmed	bibliography database http://www.ncbi.nlm.nih.gov/pubmed/
ExPASy Proteomics Server	Identification of protein domains, families and functional sites as well as associated patterns and profiles http://expasy.org/prosite/

Table 3.6: Internet resources used in this study

Aplication	Kit-Name	Producer/ manufacturer
DNA isolation	DNAeasy Blood and Tissue kit	QIAGEN GmbH
DNA quantification	Quant-it PicoGreen®-DNA-Quantitation Kit Invitrogen®	Molecular Probes
Gel extraction		
Genomic walking sequencing	DNA Walking *Speedup* ™ Premix kit	Seegene
PCR	puRe Taq Ready-To-Go Beads	GE Healthcare GmbH
PCR	PCR Master Mix	fermentas GmbH
PCR large amlicons	Expand Long Template PCR	Roche Diagnostics
PCR product purification / gel extraction	GFX PCR DNA and Gel Band Purification Kit	QIAGEN GmbH
Plasmid preparation	Plasmid Mini Kit, Midi kit	QIAGEN GmbH
Probe labeling (Southern Hybr)	DIG High-Prime	Roche Diagnostics
Sequencing of PCR products	Cycle Sequencing Kit V3.1 (Big Dye)	Applied Biosystems

Table 3.7: Kits used for different molecular approaches in this work

through a nitrocellulose filter mounted on a filter matting apparatus (Sartious). The cell concentration of the recipient culture was determined by plating 100 µl of serial dilutions ($10^{-4}, 10^{-6}, 10^{-8}$) on BHI agar overnight at 37℃. Mating was allowed by overnight incubation of the filter at 37℃ with the bacterial cells top up on a BHI agar plate. Cells were harvested from the filter by adding 1 ml of saline solution and re-suspending the bacteria with help of a glass tool. Re-suspended cells were collected on a 1.5 ml tube and centrifuged at 8,000 rpm for 5 min at 4℃. Pellets were washed once with 500 µl of saline solution, and re-suspended in 500 µl of saline solution. Serial dilutions ($10^{-1}; 10^{-2}, 10^{-3}, 10^{-4}$) of the transconjugant suspensions were prepared and 100 µl of each were plated by duplicate on selective plates, and incubated overnight at 37℃. Antibiotics can induce SOS responses that trigger horizontal gene transfer [176, 9, 88, 156]. Antibiotic-SOS induction was used in some cases by exposing the donor strain to sub inhibitory concentrations of ciprofloxacin (1mg/L) and ampicillin (8ml/L) during 3 h previous to the filter mating. Transconjugants were selected on BHI agar containing the selective marker for the donor and those for the recipient (Tables 3.9 and 3.8)

3.7 Bacterial strains

The bacterial strains used are grouped into four categories as they were used for: (a) filter mating -transfer of the *E. faecalis* PAI, (b) filter mating -transfer of the hyl_{Efm} genomic island (GI) of *E. faecium*, (c) investigation of the structure and distribution of the *E. faecium* hyl_{Efm} GI, or (d) other purposes. *E. faecalis* strains carrying the *esp* gene were used as donor, and others lacking this marker were used as recipient (Table 3.9). hyl_{Efm} is only found in *E. faecium*. Transfer of hyl_{Efm} was intended using hyl_{Efm} positive *E. faecium* strains as donor and hyl_{Efm} negative *E. faecium* and *E. faecalis* strains as seen in Table 3.8. The "nosocomial infections" unit of the Robert Koch Institute, Wernigerode branch, serves as a focal laboratory for enterococci in Germany. In general enterococcal isolates sent to the unit are pre-selected for being vancomycin and muti-resistant or with the suspicion of clonal spread (outbreak). For investigation of the hyl_{Efm}GI and its environment, a subset 39 hyl_{Efm}-positive *E. faecium* clinical isolates (15 VRE and 24 VSE) was selected originating from 11 federal states (between 1998-2007) and from different clinical laboratories or university medical centres, with different sequence type (MLST) and MLVA type to exclude copy isolates (listed in Table 3.10). hyl_{Efm}GI structure, as well as the plasmid content of all strains and localization of hyl_{Efm} and *vanA* markers were evaluated as described below in this chapter. Other strains used are listed in Table 3.11.

Strain	MLST	Resistance	plasmid content (kb)[a]
Donors			
UW2774	ST18	VAN/ERY	281, 75
UW2460	ST17	VAN/ERY	264, 235, 213, 45, 33, 10
UW5904	ST203	VAN	**188**, 68
UW5905	ST192	VAN	**188**, 68
UW3308	ST65	VAN/AMP	**206**, 156, 101, 76, 44
UW6715	ST192	ERY	314, **165**, 272, 73, 40
UW6717	ST317	ERY	384, **187**, 270, 187, 88, 40
Recipients			
E. faecium			
64/3	ST21	FUS/RAM	None
BM4105RF	ST172	FUS/RAM	None
GE-1	ST-515	FUS/RAM	None
64SS	ST21	STR/SPECT	None
E. faecalis			
OG1RF	ST1	FUS/RAM	None
JH2-2	ST8	FUS/RAM	None
UV202	ST8	FUS/RAM	None
Transconjugant			
64/3xUW2774 T-1 [b]	-	VAN/ERY/FUS/RAM	281
64/3xUW2774 T-2 to 5		VAN/ERY/FUS/RAM	variable [c]
64/3xUW2460 T-1 to 5	-	VAN/ERY/FUS/RAM	variable [c]
64SSxT2	-	VAN/STR/SPECT	not pursued

Table 3.8: Strains used in filter mating experiments for hyl_{Efm} transfer. ST, Sequence type; FUS, fusidic acid; RAM, rifampicin VAN, vancomycin; ERY, erythromycin; GEN, gentamycin (high level); PEN, penicillin; OTE, oxitetracycline, STR, streptomycin, SPE, spectinomycin. Only antibiotic resistances relevant for the transconjugants selection are presented. [a]Approximate plasmid size resolved by S1-nuclease Pulse field gel electrophoresis (PFGE) analysis. [b]Strain also used as donor and for plasmid (pLG1) sequencing. [c] For detailed plasmid content of each strain see Table 4.2. Plasmids in bold face are hyl_{Efm}- plasmids.

Strain	MLST	Resistance	*esp* present
Donor			
E. faecalis			
UW3114	ST4	ERY/OTE/STR	Yes
Recipients			
E. faecium			
64/3	ST21	RAM/FUS	No
E. faecalis			
OG1RF	ST1	RAM/FUS	No
Transconjugants			
OG1RFxUW3114 T-12 [a]		RAM/FUS/ERY	Yes
OG1RFxUW3114 T-1[b]		RAM/FUS/ERY	No
64/3xUW3114 T-10		RAM/FUS/ERY	Yes
64/3xUW3114 T-1[b]		RAM/FUS/ERY	No

Table 3.9: Strains used in filter mating experiments for *esp* transfer. ST, Sequence type; FUS, fusidic acid; RAM, rifampicin VAN, vancomycin; ERY, erythromycin; STR, streptomycin. Only antibiotic resistances relevant for this study are presented. [a]Strain OG1RFxUW3114 T-12 was used for plasmid (pLG2) sequencing. [b] Strains that acquired a 66 kb *erm*(B)-plasmid but did not acquire the *esp* PAI.

Strain	Year	MLST	MLVA	van
DO	1991	18		
UW1952	1998	18	1	vanA
UW2774	1999	18	281	vanA
UW2460	1999	17	1	vanA
UW2457	1999	80	1	vanA
UW3056	2000	65	5	0
UW3488	2001	18	1	vanA
UW3183	2001	18	1	vanA
UW3308	2001	65	-	vanA
UW5905	2004	192	159	vanA
UW5352	2004	17	7	vanB
UW5275	2004	376	1	vanB
U0317	2005	78		
UW6379	2005	18	1	vanA
UW6352	2005	18	1	vanA
UW6151	2005	17	1	vanA
UW6112	2005	378	5	0
UW6993	2006	192	159	0
UW6990	2006	18	7	0
UW6985	2006	81	7	0
UW6982	2006	192	159	0
UW6951	2006	192	159	0
UW6947	2006	17	7	0
UW6943	2006	192	159	0
UW6941	2006	192	12	0
UW6929	2006	192	159	0
UW6893	2006	192	159	0
UW6883	2006	378	new	0
UW6882	2006	117	282	0
UW6717	2006	317	-	0
UW6715	2006	192	159	0
UW6711	2006	192	159	0
UW6498	2006	18	7	vanA
UW6923	2007	202	1	0
UW6920	2007	117	282	0
UW6919	2007	192	159	0
UW6918	2007	17	7	vanB
UW6917	2007	17	1	0
UW6916	2007	378	new	0

Table 3.10: Strains used for investigation of hyl_{Efm} GI structure, hyl_{Efm} genomic location and distribution. vancomycin resistance type is shown for vancomycin resistant strains.

Strain	Description	Reference
Staphylococcus aureus NCTC8325	Size marker in PFGE	
Streptococcus equi	Hyaluronidase activity assay	
Escherichia coli EHEC	Positive control in adherence to epithelial cells.	
Escherichia coli DH5-α	Negative control in adherence to epithelial cells	
E. faecium U0317	Bears hyl_{Efm} GI	[179]
E. faecium DO	Bears hyl_{Efm} GI	[120]
E. faecium E1165	Bears the complete *E. faecium* esp PAI	[79]
E. faecalis MMH594	Bears the complete *E. faecalis* PAI	[141]
plasmids		
pLG1	hyl_{Efm} *vanA* ca. 281.02 kb from strain 64/3xUW2774 T-1	this study
pLG2	*erm*(B) ca. 66 kb from strain OG1RFxUW3114 T-12	this study

Table 3.11: Strains (other than for conjugation experiments) and plasmids used in this study.

3.8 Isolation of whole cell DNA

Genomic DNA was isolated using DNAeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) following the instructions of the manufacturer, with addition of an initial lysis step for better breakage of the cell wall. 1 ml of an overnight culture in BHI broth at 37 ℃, was centrifuged and re-suspended in 180 µl Gram positive cells-lysis buffer (not included in the kit) and incubated at 37 ℃ for 60 min. Then 25 µl ProteinaseK and 200 µl Buffer AL were added, and further incubated at 70 ℃ for 60 min. 200 µl ethanol 98% were added, mixed and the whole content added into the DNAeasy spin mini column. Columns were centrifuged at 8,000 rpm for 1 min, 200 µl buffer AW1 were added to the columns and then centrifuged again. 200 µl buffer AW2 were added and centrifuged again at 14,000 rpm for 3 minutes. DNA was eluted from the column incubating two times for 1 min with 15 µl buffer AE at room temperature and centrifuging again at 8,000 rpm for 1 min. DNA was stored at -20 ℃ avoiding thaw and freezing.

Gram positive cells-lysis buffer	
Tris, pH 8.0	20 mM
EDTA	2 mM
Triton X-100	1.2%
Lysozym	20 mg/ml (added before use)

Tube	Amount DNA	Amount	Conc.
		TE µl	DNA (μg/ml)
1	5 µl lambda DNA	95	5.0
2	50 µl from 1	50	2.5
A1	40 µl from 2	60	1.0
A2	50 µl from A1	50	0.5
A3	40 µl from A2	60	0.25
A4	50 µl from A3	50	0.1
A5	40 µl from A4	60	0.05
A6	50 µl from A5	50	0.025
A7	40 µl from A6	60	0.01
A8	-	100	0.0
B1	1 µl probe	99	unknown
B2	10 µl from B1	90	unknown

Table 3.12: Scheme for PicoGreen DNA quantification. Standard curve preparation (A) and investigated sample (B) dilutions are indicated.

3.9 DNA concentration determination

DNA average concentration was determined using a SmartSpect™ spectrophotometer (Biorad) at default meassurement conditions for dsDNA quantification (A260nm, conversion factor: 1.0=50.0 μg/ml). For more specific quantification and for plasmid samples DNA concentration was determined using Quant-iT PicoGreen®, an ultra-sensitive fluorescent nucleic acid stain for quantification of double-stranded DNA (dsDNA) in solution. A standard curve using the provided lambda DNA was prepared for every measurement, as well as appropriate dilutions of the samples as shown in Table 3.12.

A solution 1:200 of PicoGreen in TE was prepared and 50 µl of this solution was added into each flat-bottomed well of polystyrene black microtiter plates. 50 µl of each dilution A1 until A8 and B1 until Bx were diluted into the 50 µl of PicoGreen solution into the wells. The microtiter plates were scanned on a florescence reader at 480-520 nm.

3.10 PCR

3.10.1 Regular PCR

The PCR reactions were performed using PuReTaq Ready-To-Go PCR Beads (GE Healthcare Europe GmbH) which are dried beads containing Taq Polymerase, nucleotides and buffer. Water, primers and template were added to final volume of 25 µl which contained1.5 U Taq DNA polymerase, 200 µM each dNTP, 0,1 µl template

MM 1:	Final conc.:	**MM2**:	Amount:
dNTP each	500 µM	PCR Buffer 10x	5 µl
Primer each	400 nM	Expand long	
Template DNA	300 ng	Enzyme mix	0,75 µl
Water	up to 25 µl	Water	up to 25 µl

Table 3.13: Long PCR reaction setting.

DNA. Cycling conditions were variated according to the annealing temperature of the used primers and expected amplicon size, but in general were as follows: after a denaturing step, 35 cycles were run for 30 s at 94 ℃, 30 s at 50 ℃ and 30 s at 72 ℃ followed by a final step of 5 min at 72 ℃. The PCR products were resolved in 1.0% agarose gels, these were stained with ethidium bromide for 15 min, rinsed for other 15 min and visualized in UV light.

3.10.2 Long template PCR

Long template PCR was performed using an Expand long template PCR (Roche) under the conditions recommended by the manufacturer as follows: two master mixes (MM) were prepared separately and then mixed to a 50 µl final volume as presented in Table 3.13.

Cycling conditions included increased extension times to ensure a higher yield of amplification product. After an initial denaturation step at 92 ℃ for 2 min, 10 cycles were run for 10 s at 92 ℃, 30 s at 50-65 ℃, 10-20 min (elongation) at 68 ℃. Additional 20 cycles were run for 15 s at 92 ℃, 30 s at 50-65 ℃, 10-20 min (elongation) at 68 ℃ plus 20 s cycle elongation for each successive cycle. The final elongation cycle was for 7min at 68 ℃. The elongation time used was 10 min for PCR products between 5 and 10 kb and 15 min for larger expected products.

3.10.3 Determination of *E. faecalis* PAI and putative hyl_{Efm} GI presence and structure

The GI containing hyl_{Efm} previously suggested by Rice *et al.* [120] was searched among the available *E. faecium* genome sequences of strain DO (NZ_AAAK00000000) and U0317 (NZ_ABSW00000000) and the different sequences found were compared by BlastN and re-annotated into one sequence with the help of DSgene and Kodon software. The *E. faecalis* PAI structure described by Shankar *et al.* [141] $gb|AF454824.1|$, was used as a reference for the investigation of PAI structure.

Transconjugants were tested for co-transfer of *esp* gene by PCR using the primers esp1 and esp2 and for co-transfer of the hyl_{Efm} gene using the primers hyl1 and hyl2 (Table A. 1). For the screening of *esp* positive transconjugants, pools of genomic DNA of 20 colonies were analyzed in each PCR reaction, and if positive, single

colonies were investigated in detail. (see table A. 1).

Presence of the whole *E. faecalis* esp PAI was tested by amplification of overlapping regions of ca. 10 kb by long template PCR (PAI regions 1 to 9). Regions yielding unexpected size or no product were further investigated by regular PCR of each predicted ORF present into that region. Primers used are listed in table A. 1. Presence of the whole hyl_{Efm} GI was tested by amplification of three overlapping regions (hylGI1, hylGI2 and hylGI3) of ca. 5.5 kb by long template PCR. Region hylGI3 was analysed by amplification of smaller regions (hylGI3a, hylGI3b, hylGI3c, hylGI3d) for further detailed analysis. Presence of replication, mobilization and maintenance genes of the hyl_{Efm} plasmid pLG1 was investigated by regular PCR using the primers listed in Table A. 1 (rep-, MobC-, rel-, repB-, MazE-pUW2774). All the primers used for hyl_{Efm} genomic environment are listed in Table A. 1. Specificity of the PCR products was confirmed by HindIII digestion. The digestion reaction was set with 1 µl of each PCR product, 40 units of the enzyme (2 µl), 1.0 µl of NEBuffer2, and 6 µl H_2O, and was incubated overnight at 37℃. Prediction of the digestion fragment sizes was done using NEBcutter V2.0 (`http://tools.neb.com/NEBcutter2/index.php`).

3.10.4 Detection of excision, circularization and integration of the *E. faecalis* PAI

Primers binding outside of the integration site of the esp PAI PAI164 / PAI167 and PAI164Efm / PAI167Efm were used to amplify the intact integration site in the *E. faecalis* and *E. faecium* strains respectively. Primers EF2 and ER1 binding inside the PAI (reading outwards the PAI) were used in different combinations with primers binding outside of the PAI to demonstrate the integration site in the transconjugants. Excision of the *E. faecalis* PAI from the chromosome of *E. faecalis* was tested by two stage nested PCR with primers amplifying the integration site of the PAI, under the following conditions: after a denaturing step, 35 cycles were run for 30 s at 94℃, 60 s at 55℃ and 60 s at 72℃ followed by a final step of 5 min at 72℃. For the first round the primers PAI164 and PAI167 were used, and for the second round 1 µl of PCR product was used for amplification of an inner region thereof using primers PAI164nes and PAI167nes (Table A. 1, Figure 3.2). Circularization of the *E. faecalis* PAI were tested by two-stage nested PCR with primers binding in the *E. faecalis* PAI ends which are supposed to join during circularization, and reading outwards the PAI under the following conditions: after a denaturing step, 35 cycles were run for 30 s at 94℃, 30 s at 55℃ and 60 s at 72℃ followed by a final step of 5 min at 72℃. For the first PCR the primers TSP2 5' and TSP2 3' were used, and for the nested PCR 1 µl of PCR product was used for amplification of an inner region thereof using primers TSP3 3' and TSP3 5'. (Table A. 1, Figure 3.2). All PCR products were sequenced.

3.11 Genomic walking

The DNA Walking *Speedup* ™ Premix kit (Seegene) was used for investigation of the unknown regions adjacent to a known DNA region. This genomic walking technique employs Annealing control primers (ACP) which enhances specificity of short oligomers making DNA walking experiments more simple and cost-effective. The kit is composed of a PCR master mix and unique DNA Walking-Annealing Control primers (DW-ACP™) that are designed to capture unknown targets with high specificity. The optimized PCR conditions (referred to as DW ACP-PCR technology) allow unknown flanking regions of up to 3 kb long to be obtained. One of the four ACP primers and the target specific primer 1 (TSP 1) were used to amplify the target region from the template in the first PCR. The first PCR product was purified using a QiaQuick PCR purification kit. The second PCR (the first nested PCR) used the DW-ACPN primer and the TSP 2 primer to amplify the target from the first PCR product. The third PCR (the second nested PCR) used Universal primer and TSP 3 and the second PCR product as a template (see figure 3.1). The Specific primers used were designed according to the specifications of the manufacturer. For investigation of the genomic region flanking the *E. faecalis* PAI in *E. faecium*, following primers were used TSP1 5', TSP2 5' and TSP3 5', tagging the 5'end of the PAI and TSP1 3'TSP2 3', TSP3 3' tagging the 3' end of the PAI (see table A. 1). To investigate the flanking regions of the hyl_{Efm} GI primers TSP1hylGI 3', TSP2hylGI3' and TSP3hylGI 3', tagging the 3' end and primers TSP1hylGI 5', TSP2hylGI 5', TSP3hylGI 5' tagging the 5' end were used. The PCR products obtained were visualized on 1% agarose gels, bands were extracted and sequenced directly using both the universal primer and the corresponding TSP3 primer. The PCR conditions were s shown in Table 3.14.

3.12 Sequencing

PCR products were completely or partially sequenced according to the manufacturer recommendations for cycle sequencing of PCR products (Applied Biosystems). A sequencing reaction was set as shown in Table 3.15. The cycling conditions were as follows: after a 2-minutes denaturing step at 96 ℃, 25 cycles were run for 10 s at 96 ℃, 5 s at 45 ℃ to 60 ℃ and 4 min at 60 ℃.

First PCR				
Reagents		Cycling conditions		
Genomic DNA	50-100 ŋ	94 ℃	5 min	1 cycle
2.5 µM each DW-ACP		42 ℃	1 min	
(DW-ACP 1, 2, 3, 4)	4 µl	72 ℃	2 min	
10 µM TSP1	1 µl	**94 ℃**	**40 s**	**25 cycles**
2x SeeAmp ACP		**55 ℃**	**40 s**	
Master Mix II	25 µl	**72 ℃**	**90 s**	
H_2O	x	72 ℃	7 min	1cycle
Total	50 µl	4 ℃	∞	
Second PCR				
Reagents		Cycling conditions		
Purified first PCR product	3 µl			
10 µM DW-ACPN	1 µl	94 ℃	5 min	1 cycle
10 µM TSP2	1 µl	**94 ℃**	**40 s**	**30 cycles**
2x SeeAmp ACP		**60 ℃**	**40 s**	
Master Mix II	10 µl	**72 ℃**	**90 s**	
H_2O	5 µl	72 ℃	7 min	1cycle
Total	20 µl	4 ℃	∞	
Third PCR				
Reagents		Cycling conditions		
second PCR product	1 µl			
2.5 µM Universal Primer	1 µl	94 ℃	5 min	1 cycle
10 µM TSP3	1 µl	**94 ℃**	**40 s**	**30 cycles**
2x SeeAmp ACP		**60 ℃**	**40 s**	
Master Mix II	10 µl	**72 ℃**	**90 s**	
H_2O	7 µl	72 ℃	7 min	1cycle
Total	20 µl	4 ℃	∞	

Table 3.14: DNA-Walking PCR setting scheme (Seegene)

Reagent	Final conc.:
PCR-Product (200-500 bp)	3-10 n*g* or
PCR-Product (500-1 kb)	10-20 n*g*
Primer	5 pmol
BigDye 3.1	1 µl
H_2O	up to 10 µl

Table 3.15: Sequencing reaction setting

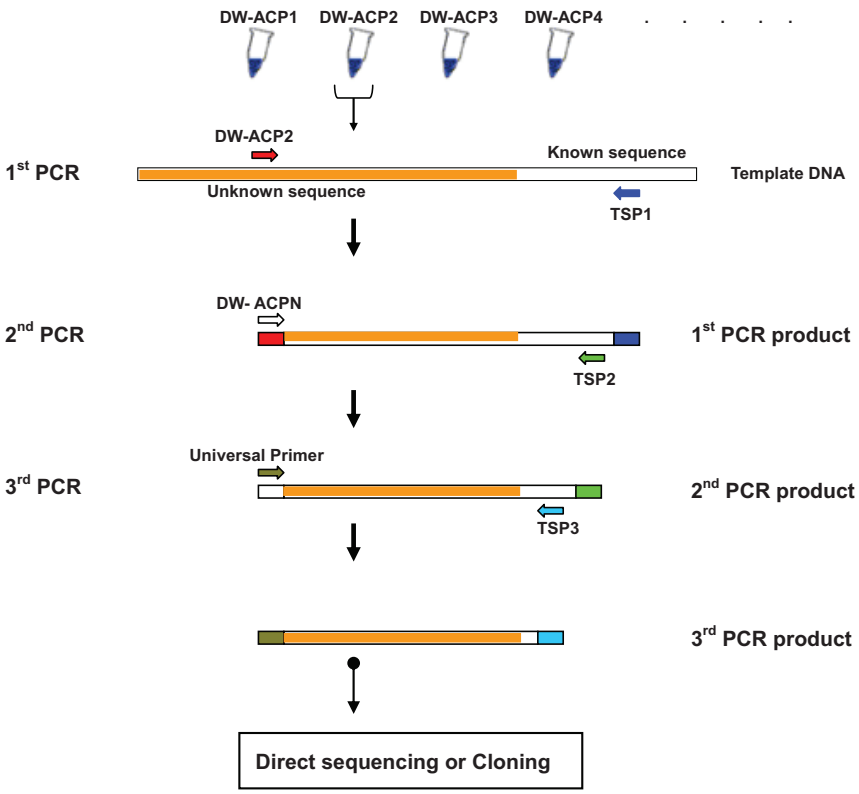


Figure 3.1: The general strategy of DNA walking ACP™ PCR technology. DW-ACP (DNA Walking Annealing Control Primer); TSP (Target Specific Primer). Source: Seegene, Inc SpeedUp ^{TM} User's manual

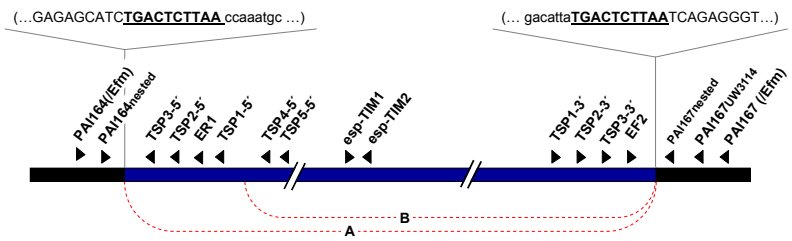


Figure 3.2: Representation of the *E. faecalis* PAI showing the primers used in this study for *esp* detection and investigation of PAI excision, circularization and integration (not to scale). Black arrowheads represent the primers. Solid black region represents chromosomal DNA, grey region represents the PAI. The junction- nucleotide sequences after PAI integration in the chromosome of *E. faecalis* are shown in detail; the direct repeats (DR) appear in underlined bold text. Dashed lines indicate joining of PAI regions during formation of circular intermediates; A and B indicate two different events of excision and circularization of the PAI that were confirmed in this study (see Results and Discussion sections).

3.13 Pulse field gel electrophoresis (PFGE) and southern hybridization

The Pulse field gel electrophoresis (PFGE) can resolve macrorestriction fragments of DNAs and can be used to type microorganisms at the molecular level. PFGE involves embedding of the organisms in agarose, lysing the organisms in situ and treating with enzymes and detergents to remove all cellular components from the DNA. Finally the total DNA is digested with restriction endonucleases that cleave the DNA infrequently (*Sma*I for enterococci). The restriction fragments (10 to 20 for enterococci with sizes varying between 5 and 400 kb for *E. faecium*, 600 kb for *E. faecalis*) are separated in an agarose gel by alternating the electric field between spatially distinct pairs of electrodes arranged in a hexagonal contour. The varying electric field allow the separation of DNA fragments ranging in size from a few kb to some megabases. Preparation of samples and subsequent macrorestriction was done as previously described [197, 192] For DNA -preparation, cells were grown in 5 ml of triptic soy broth (TSB) overnight at 37℃. Cells were harvested from 1.0 ml of this culture and suspended in PIV buffer. The cells were transferred to an Eppendorf tube, centrifuged and resuspended in 1 ml of ice-cold PIV. 50 µl of this suspension were mixed with 50 µl of 1.2% LMP agarose and immediately pipetted into the sample mold (BIO-RAD) and allowed to solidify. For lysis each plug was placed in 1 ml of lysis buffer and incubated overnight at 37℃ under gentle shaking. The plugs were washed twice with 1 ml ES-buffer and incubated in 1 ml ESP-buffer overnight at 50 ℃. The plugs were washed three times for at least 30 minutes at room temperature with 14 ml of TE-buffer. For digestion with *Sma*I the agarose plugs were first given 1 ml 1x *Sma*I-buffer for 30 min to equilibrate, and then freshly prepared 1x *Sma*I buffer with 40 U *Sma*I enzyme per plug. using a CHEF-DR III apparatus (Biorad). The sizes of the restriction fragments were predicted according to an external size standard (*Sma*I digested *Staphylococcus aureus* NCTC8325) and calculations using software BioNumerics v. 5.1 (Applied Maths) [98].

Lysis-buffer

Tris-HCl pH 7.6	6 mM
Sodium chloride	1 M
Disodium EDTA pH7.6	100 mM
Brij 58	0.5%
Sodium deoxycholate	0.2%
Sodium lauroylsarcosine	0.5%
pH 7.6	
Add before use:	
RNAase	60 µg/ml
Lysozyme	1 mg/ml

ES-buffer

Disodium EDTA pH 9.0	5.0 M
Lauroylsarcosine	1%
pH 9.0	

PIV-buffer

Tris-HCl pH 8.0	10 mM
Sodium chloride	1 M
pH 7.6	

LMP-agarose

1.2% agarose in PIV-buffer

ESP-buffer

Proteinase K 1 mg/ml in ES-Buffer

TE-buffer

Tris-HCl	10 mM
Disodium EDTA	1 mM
pH 7.5	

3.13.1 Southern blot

Southern-blot is a method for transferring DNA fragments from agarose gels to solid supports (nylon membranes or nitrocellulose filters). The nylon membranes are positively charged for a more efficient binding of the negatively charged DNA to the membrane. The methods used for southern hybridization were capillary transfer and vacuum transfer of DNA to positively charged nylon membranes according to the methods described by Sambrook *et al.* [132]. Capillary transfer involved the transfer of DNA from the gel on the surface of nylon membranes, carried on a flow of liquid which is drawn through the gel by capillary action established by a stack of dry absorbent paper towels. Capillary transfer was used for transfer of DNA from macrorestriction analysis resolved in pulse field gels. Prior to the transfer the gels were washed in different buffers in order to render the DNA in smaller single-stranded fragments, which are then more efficiently eluted from the gel. The gel was washed as follows: once for 10 min in Blot-buffer 1, 2 times 15 min in Blot-buffer 2, and 2 times 15 min in Blot-buffer 3. A dish was filled with the transfer buffer and the blot was built as follows: the inverted gel was placed on wet 3MM Whatman paper whose ends were immersed in the transfer buffer; over the gel the membrane (1 mm larger than the gel in both dimensions) was placed and on the membrane two pieces of 3MM Whatman paper and a stack of dry paper towels cut of the same size of the membrane. The transfer was allowed to proceed for 12 - 18h. To fix the DNA to the membrane the side of the membrane carrying the DNA was crossed linked by ultraviolet irradiation exposure (150 mJoule, 30 s). The membrane could then be used for hybridization experiments.

Vacuum transfer was used for rapid transfer of DNA from gels to nylon mem-

branes, and was used for the transfer of DNA from plasmid preparation, PCR and S1 nuclease gels. The transfer method was performed according to the instructions accompanying the Vacuum Blotter device (Apligene). The blot was constructed placing the membrane on the blot surface under the gel. The transfer was performed at 50 mbar. The buffers were poured on the gel covering it completely. The gel was washed as follows: Blot-buffer 1 for 30 min, Blot-buffer 2 for 30 min, vacuum-Blot-buffer 3 for 30 min. Finally the transfer buffer was given for 120 min on the gel. The wet membrane was crossed linked by ultraviolet irradiation exposure (150 mJoule, 30 s).

Blot buffer 1	
HCl	0.25 M
Blot buffer 2	
NaOH	0.5 M
NaCl	1.5 M
Blot buffer 3	
Tris	0.5 M
NaOH	20 mM
Transfer buffer	
NaCl	3 M
Sodium citrate	0.3 M
Vacuum blot buffer 3	
Tris	1 M
NaCl	2 M

3.13.2 Probe labelling

Digoxigenin (DIG)-11-dUTP alkali-labile was used to label DNA probes using PCR DIG-High Prime system (Roche), according to the random primer labelling technique. 1 µg of DNA to be labelled (i.e. PCR products) were diluted in 16 µl of H_2O. The DNA was denaturised for 10 min at 96 ℃ and immediately cooled in ice. 4 µl of High-Prime solution were added and incubated for 2 h at 37℃. The reaction was stopped by heating at 65℃ for 10 min. *esp* probes were generated using primers esp1 and esp2; *erm*(B) probes, using primers ermB1 and ermB2; hyl_{Efm} probes, using primers hyl1 and hyl2; *vanA* probes using primers vanA1 and vanA2, pilA probes using primers piliF and piliR. Other probes were produced using the primers listed in table A. 3.

3.13.3 Southern hybridization

Southern hybridization was done using DIG system kits following the manufacturer's recommendations (Roche). The Southern blot membranes loaded with single-stranded DNA were hybridized with a DIG-labelled DNA probe. The nylon membranes were pre-hybridized with 20 ml hybridization buffer at 37℃ for 2 h. The solution was replaced with 12 ml hybridization buffer containing 5 µl freshly denaturised probe DNA and was incubated for 12 h at 37℃. The membrane was then washed twice with SSC-buffer 1 for 5 min and twice with SSC-buffer 2 for 15 min. After this the membrane could be used for immunological detection.

20 x SSC buffer	
NaCl	3 M
NaCitrat (pH7)	0.3 M
Hybridization-buffer 1	
SSC	5 x
Blocking reagent	1 x
N-lauroylsarcosine	0.1%
SDS	0.02 %
SSC-buffer 1	
SSC	2 x
SDS	0.1 %
SSC-buffer2	
SSC	0.1x
SDS	0.1 %
Blocking reagent (Stock solution)	
Blocking reagent (Roche) 10% in Hybridization-buffer 1	

3.13.4 Immunological detection

Immunological detection was performed using the DIG nucleic acid detection kit (Roche). DIG-labelled DNA was detected after hybridization to target nucleic acids, using an antibody-conjugate (anti-DIG alkaline phosphatase conjugate, anti-DIG-AP) which binds selectively to the DIG-labelled d-UTP (probe). The complex formed is then visualized with CDP-Star (Roche), a chemi luminescent substrate for alkaline phosphatase. The membranes were washed briefly in Detection-buffer 1 + 0,3%Tween® 20, then incubated for 30 min with 100 ml Detection buffer 2. Then the membranes were incubated for 30 min with 50 ml of diluted antibody conjugate solution 1:10,000 in Detection-buffer 2. The unbound antibody conjugate was removed by washing twice for 15 min with 100 ml of Detection-buffer 1 and the Membrane was

equilibrated for 2 to 5 min with 20 ml of Detection-buffer 3. The membrane was then placed into a hybridization bag where 7 µl CDP-Star in 1 ml Detection-buffer 3 were given and left for 5 min to react protected from the light. The CDP star solution was removed. Visualization of hybridized fragments was done using a Biodocumentation luminescence detector ChemiDoc X R (Biorad) by extended exposure of at least 1 h.

Detection-buffer 1	
Maleic acid	0.1 M
NaCl	0.15 M
pH 7.5	
Detection-buffer 2	
blocking reagent (Roche)	1x
Detection-buffer 1	9x
Detection-buffer 3	
Tris-HCl	0.1 M
NaCl	0.1 M
MgCl2	50 mM
pH 9.5	

3.13.5 I-*Ceu*I PFGE

Chromosomal location was analyzed by southern hybridization of I-*Ceu*I macrorestriction analysis as previously described [110]. I-*Ceu*I is an intron-encoded endonuclease, used to cut a 26 bp site in the rRNA genes, coding for the 23S large subunit rRNA The agarose blocks containing total bacterial DNA were prepared for restriction as previously described [192]. Previous to restriction, the blocks were equilibrated in 100 µl of a 20 mM Tris pH 8.0 5 mM EDTA solution for 30 minutes at room temperature. This solution was replaced for 100 µl of a second equilibration solution containing 1 µ*g* BSA, 20 µl 10x enzyme buffer NEB4 and 79 µl water, and were incubated for 30 min at 4 ℃. Finally the blocks were incubated overnight in a lysis solution containing 4 U of I-I-*Ceu*I, 1 µ*g* BSA 10 µl NEB4, and 88,2 µl water. The ramped pulse field times were as follows:5.0-30.0 s for 23 h at 14 ℃. *Sma*I digested *S. aureus* NCTC8325 was used as the size indicator.

3.13.6 S1-nuclease PFGE

Plasmid content analysis and possible plasmid localization was evaluated by southern hybridization of S1-nuclease macrorestriction analysis by the method described by Barton *et al.* S1-nuclease is a unique enzyme in that it is effective on any super coiled plasmid, has a self limiting action and makes few cleavages in chromosomal DNA. These features are consequences of its specificity for unpaired nucleic acids.

In negatively super helical plasmids, transiently denaturated regions sensitive to S1 action occur as a result of torsional stress in the molecule. After one strand is cut, S1 can cleave the intact strand opposite the initial break due to "breathing" of ends at the nick where the cooperativity of base pairing is diminished. Under this conditions, the enzyme has little subsequent activity on linear, double stranded DNA. S1 probably cuts supercoiled domains in genomic DNA but the cleavages are sufficiently infrequent to produce relatively few fragments. Consequently, linearised, single-copy plasmids are detectable after electrophoresis as bands in a faint genomic background [8]. The agarose blocks containing total bacterial DNA were prepared for restriction as previously described [192]. The agarose blocks were equilibrated in 100 µl tris-HCl, 10 mM pH 8, then incubated for 15 min at 37 ℃ in 100 µl of enzymatic solution containing 14 Units S1-nuclease (Takara) and 10 µl of 10x S1-nuclease buffer. Finally the enzymatic solution was carefully removed and blocks were equilibrated for 1 h in 100 µl TE buffer. The ramped pulse field times were as follows:5.0-35.0 s for 22 h at 14 ℃. *Sma*I digested *S. aureus* NCTC8325 was used as the size indicator.

3.14 Plasmid isolation

Plasmids were isolated after modification of the method from Woodford *et al.* as follows: Cells were grown on BHI agar plate at 37 ℃, re-suspended in 1ml TES buffer, pH 8.0 and centrifuged at 8,000 rpm for 5 minutes. Pellets were re-suspended and incubated during 60 min at 37 ℃ in 200 µl TES buffer containing 10 mg/ml lysozyme and 5 μg/ml RNAase. 400 µl 0,2 N NaOH / 1% SDS were added, the tube was mixed gently and further incubated at 56 ℃ for 60 min. 300 µl 3 M potassium acetate, pH8,4 were added and the tube was kept on ice for 20 minutes, following centrifugation at 15,000 rpm for 15 min. The supernatant was carefully transferred into a new tube. then the supernatants were washed 3 times by adding 3 volumes of Phenol / chloroform / isoamyl alcohol (25:24:1), inverting it ca. .30 times and centrifuging it at 12,000 rpm for 10 minutes and collecting the upper, aqueous phase. One extraction using only Chlorophorm followed. After this the DNA was precipitated overnight at -20 ℃ with 2 vol. ethanol 98%, following a final centrifugation at 15,000 rpm for 30 min at 4 ℃. The pellets were washed with 500 µl 70% ethanol and centrifuged, then dried at 37 ℃ during 30 min and dissolved in 20 µl water for 20 min at room temperature. The final DNA concentration was determined using PicoGreen and the plasmid preparation was visualized by electrophoresis in 1% agarose gel.

3.15 454-sequencing

454 Sequencing is a large-scale parallel pyrosequencing system capable of sequencing approximately 100 megabases of DNA per 10-hour run on the Genome Sequencer FLX with GS FLX Standard series reagents. The technology is known for its unbiased sample preparation and long, accurate sequence reads (250 base pairs in length), including paired reads.

Two plasmids were sequenced by means of 454 technology at GATC Biothech (Konstanz, Germany):
(a) pLG1: transferable megaplasmid (ca. 281.02 kb) bearing hyl_{Efm} and *vanA* from transconjugant strain 64/3xUW2774 T-1.
(b) pLG2: transferable plasmid (ca. 66 kb) from transconjugant OG1RFxUW3114 T-12, strain that acquired the *esp* pathogenicity island. The plasmid sequencing at GATC Biotech was done according to standard procedures for sequencing on the Genome Sequencing (GS) FLX system (Roche).

For preparation of the single read libraries the double stranded DNA (dsDNA) was column purified and sheared on a Covers ATA to fragments in a size of ca. 700 bp. The A and B adapters for sequencing of the Roche technology were ligated to the ends of the sheared DNA fragments. The samples were run on a 2% agarose gel and the band in a size range of 700 to 900 bp was excised and column-purified. After concentration measurement the resulting library was immobilized onto DNA capture beads and the amplicon-beads obtained were amplified through emPCR according to the manufacturer's recommendations. Following amplification the emulsion was chemically broken and the beads carrying the amplified DNA library were recovered and washed by filtration. The samples were sequenced on a GS FLX Pico-Titer plate device.

3.16 Sequence analysis and annotation

All sequences were analysed using DNAStar software SeqMan Engine and DS Gene software packages, as well as BlastX and BlastN. After 454 plasmid sequencing, preassembled contigs were obtained. Southern hybridization allowed confirmation of plasmid localization of contigs that contained chromosomal-like sequences in pLG1. The primers used for probe synthesis are listed in Table A. 3. Gap closure was done when possible in pLG1 sequences by PCR, long PCR, primer walking and cycle sequencing. The primer pairs used for gap closure are listed in Table A. 4. putative coding sequences (CDS) were predicted and annotated using Kodon® and with the help of BLAST programs. Open reading frames equal or greater than 100 bp with initiation codon were searched, deleting smaller features with an overlap larger than 50% with a feature size less than 50% of the bigger feature size. Feature annotation of CDS was accepted with an identification score over 70%. Identification of origin

of replication, origin of vegetation, and other conserved motifs was done with the help of BlastX, BlastN and visual inspection. Phylogenetic analysis of the replicase and relaxase genes of pLG1 was done according to Weaver *et al.* and Garcillan-Barcia *et al.*. The RepA homologs, retrieved from the NCBI database by a standard BLAST search using pAD1 RepA as query, were analyzed together with repA-pLG1. The un-rooted phylogenetic tree was drawn using the CLC Combined Workbench sequence analysis software at default settings [185]. The relaxase homologs retrieved from the databases using PSI-BLAST and iterative BLAST searches were analyzed together with the rel-pLG1. The phylogenies were constructed with MEGA version 3.1. A phylogenetic tree was built using neighbour-joining (NJ) analysis, tested with bootstrap values (1000 replicates) [40].

3.17 Hyaluronidase activity

Two different tests for evaluation of hyaluronidase activity were used. For the establishment of the tests *S. aureus* NCTC8325 and NCTC8325-1 were used as positive controls and *E. faecium* strain 64/3 was used as a negative control. The first is a simple method used for routine-detection of hyaluronidase activity in pneumococci (van der Linden, M. and Nicklas, W. personal communication). A strain of *Streptococcus equi* which produces an external capsule made mainly of hyaluronic acid and does not produce hyaluronidase, was used. Each test strain (PCR hyl_{Efm} positive strains) was inoculated onto agar plates together with the *S. equi* strain in crossed streaks and was incubated for 24 or 48 hours at 37℃ and 40℃. Degradation of the mucous capsule produced by *S. equi* was observed in the region where a hyaluronidase positive strain had grown. The second is a plate method using BHI agar, supplemented with Bovine serum albumin (BSA) (1% w/v) and hyaluronic acid sodium salt from *S. equi* (0.04% w/v) [154, 35]. Cells were inoculated onto hyaluronic acid agar plates and were incubated during 24 and 48 hours at 37℃ and 40℃. Undegraded hyaluronate precipitated around grown bacteria as a conjugate with albumin in the presence of 2M acetic acid for 15 minutes. Hyaluronate lyase activity was observed as a clear zone against a background precipitate.

3.18 Transmission immunoelectron microscopy

Transmission immunoelectron microscopy was used to visualize the Esp protein and pilA pili expressed on the bacterial surface as previously described [180, 59] Bacteria were grown on Mueller Hinton (MH) blood agar plates at 37℃ and re-suspended in PBS (1×10^9 CFU/ml. A drop of re-suspended bacteria was placed on Parafilm, and a 200-mesh Formvar-carbon-coated copper grid was floated on the surface for 10 min. Grids were washed three times by floatation for 5 min on drops of 0.02 M glycine

in PBS and blocked by floatation for 15 min on drops of 1% bovine serum albumin in PBS. Esp was labelled by floating the grids for 1 h on drops containing a 1/100 dilution of anti-Esp rabbit immune serum in PBS. Grids were washed four times by floating them for 2 min on drops of 0.1% bovine serum albumin in PBS. Antibodies were labelled by floating the grids for 20 min on drops of protein A-Gold (15 nm) in PBS. Grids were washed by floatation four times for 2 min on drops of PBS, fixed by floatation on drops of 1% glutaraldehyde, and washed again by floatation eight times for 2 min on drops of H_2O. Bacteria were stained by floating the grids for 5 min on drops containing 1.8% methylcellulose (25 centipoises) and 0.4% uranyl acetate (pH 4) and subsequently air dried for 10 min. Grids were examined by using a Jeol 1010 transmission electron microscope.

3.18.1 Flow cytometry

Flow cytometry was used for quantitative detection of Esp on the bacterial surface as previously described [180]. For flow cytometry assays bacteria were grown over night on MH blood agar plates, and re-suspended in RPMI 1640 containing 0.05% human serum albumin (HSA) to an OD660 of 1.0 (1×10^9 CFU/ml). 100 µl of each sample were pelleted by centrifugation (1 min ×8000 rpm), and re-suspended in 50 µl RPMI 1640-HSA containing 1:100 anti esp-rabbit immune serum. Bacteria were incubated 30 min on ice and then washed with 1 ml cold RPMI 1640-HSA. Cells were centrifuged (1 min ×8000 rpm) and re-suspended in 50 µl RPMI 1640-HSA containing 1:50 goat anti-rabbit flourescein isothiocyanate. Samples were left for 30 min on ice, then 1 ml cold RPMI 1640-HSA was added and cells were centrifuged (1 min × 8000 rpm). Bacteria were re-suspended in 50 µl RPMI 1640-HSA. Before analyses in a FACSCalibur flow cytometer, bacteria were re-suspended in 300 µl RPMI 1640-HSA. All measurements were performed with the same machine using the same parameters. The data were normalized for bacterial size, and experiments were performed three times. The mean fluorescence (mean fluorescence channel 1) was used as a measure for cell surface-associated Esp. Each sample was incubated without anti-Esp rabbit immune serum and was used as negative controls. The specificity of the anti-Esp rabbit immune serum had been previously demonstrated by blocking the binding of serum to Esp on the bacterial cell surface after pre-incubating 1/300-diluted anti-Esp serum in RPMI 1640-HSA with serial dilutions of rN-Esp, starting with 100 µg/ml, for 15 min prior to incubation with the bacteria [180]

3.19 Biofilm formation

In vitro biofilm formation was evaluated on polystyrene microtiter plates following the methodology previously described [56] with slight modifications. Briefly,

bacteria were grown on MH blood agar plates and re-suspended in TS broth supplemented with 0.25% glucose to a concentration of 5×10^8 CFU/ml.A 100 µl aliquote of bacterial suspension was added in triplicate per well, using Flat bottomed 96-well polystyrene microtiter plates (Greiner Bio One and Corning) After incubation at 37℃ during 24 hours, the bacteria were removed and the wells were washed with 200 µl of PBS. The plates were dried for 1h at 60℃ and stained with 100 µl of 0.2% Gram's crystal violet solution (Merk, Darmstadt, Germany) during 15 minutes at room temperature. The dye was removed and the wells washed again with 200 µl PBS three times. The plates were dried 10 minutes at 60℃. The dye was re-suspended for 30 minutes by adding 100 ml of ethanol 98% into each well. Absorbance at 595 nm was measured with a Sunrise ELISA reader.

3.20 Cytolysin/haemolysin

The haemolytic activity of the strains was detected using MH blood agar plates, as elsewhere described [155]. Strains were streaked onto agar plates containing BHI agar with 5% (w/v) defibrinated human erythrocytes, 1% (w/v) glucose and 0.03% (w/v) L-arginine.

3.21 Growth

Differential growth of the strains was tested by measuring the turbidity as optical density (OD-600), produced by cells grown in BHI broth in agitation at 37℃. A Novaspec spectrophotometer was used. An inoculum of each strain was prepared inoculating a colony in 4 ml of BHI broth, growing it overnight and freezing it at -20℃. Later a 50 µl aliquot (10^8 cells/ µl) of the inoculum was added to 2,45 ml of BHI broth and OD-600 was measured every 60 minutes until the stationary phase was reached. Each experiment was repeated three times. The OD at every time point of each recipient and its corresponding transconjugant strain were compared using an unpaired two-tailed t-test.

3.22 Animal experiments

The pathogenic potential of strains was evaluated in mouse bacteraemia and peritonitis models as described previously [66, 166]. The animal studies described in the present study were reviewed and approved by the Institutional Animal Care and Use Committee at Harvard University. For preparation of the inocula bacteria were grown in 1L Todd-Hewitt broth (THB) (Bacto™) overnight, centrifuged, and re-suspended in sterile saline. Aliquots were shock-frozen and stored at -80℃. The concentration of the stock was verified by dilutions and viable counts on Triptic Soy

Agar (TSA), and these numbers were used to calculate the appropriate dilutions for the desired inoculum. For the experiments, aliquots were thawed and diluted in sterile saline solution; the actual diluted inoculum was once more verified by viable counts. Eight female 6-8-week-old BALB/c mice were inoculated intravenously in the tail vein (bacteraemia model) or intraperitoneally (peritonitis model) with 8 or 5 x 10^8 CFU respectively of each strain. 24 h after infection, the mice were sacrificed and exsanguinated, and bacterial counts in the blood and in the kidneys were done by serial dilutions. Multiple comparisons were done using one way ANOVA (PRISM4, GraphPad software).

3.23 Adherence to epithelial cells

The ability of enterococci as a gut commensal to adhere to colonic epithelium is involved in the establishment of infections since it is supposed be the first step for biofilm formation and for translocation across the intestinal barrier. Adherence of bacteria to human epithelial colorectal adenocarcinoma cells (Caco-2) was investigated. C2Bbe1 (Caco-2 Brush Border Expressing (clone) 1), ATCC CRL-2102 cells were cultivated in Dubelco's Modified Eagle's Medium (DMEM) without supplements and grown in a 5% CO_2 atmosphere. All the experiments were performed on cells between 15th to 25th passage. Cells were incubated into 24-well plates (Greiner Bio One) to a cell density of $1 - 1.2 \times 10^5$ cells/well. An overnight culture of the bacterial strains to be tested was prepared. To make a mid log phase culture 150 µl of the overnight culture were inoculated into 3 ml of BHI broth, and were incubated for 3 hours at 37℃ at 50rpm. Bacterial density was determined by measuring absorbance at 595nm in an Eppendorf Biophotometer. The Caco-2 cells were incubated for 2 h at 37℃ 5% CO_2 with bacterial suspensions in DMEM with a cell: bacteria ratio of 1:100 (1×10^7 bacteria/well). The bacterial inoculums were quantified by plating serial dilutions on BHI agar. After infection, epithelial cells were washed 4 times with PBS to remove non adherent bacteria, and were lysed with 500 µl 0.25% Triton-X at 37℃ for 5 min. Total attached and internalized bacteria were quantified by cultivation of serial dilutions of the cell-culture lysates. Bacterial quantification was done with the help of an automated spiral plater (WASP2) and a colony counter (aCOLyte). The percentages of adherent bacteria were calculated and corrected to a partial derivated error. Differences among strains were compared using an unpaired two-tailed t-test.

3.24 Statistical analysis

Data from *in vitro* growth and biofilm forming capacity of the recipient strains Vs. their corresponding PAI positive strains were compared using one way ANOVA

followed by Tukey's multiple comparison test. Results from FACS were analyzed using two way ANOVA to compare statistical significance of temperature on *esp* expression; One way ANOVA and Tukey's post test were applied to compare the *esp* expression of the different strains at 37°C. Data from animal experiments were analyzed applying Kruskal-Wallis test followed by multiple comparissons using Dunn's post-test. All statistical analysis were done using GraphPad Prism5 software.

4. Results

4.1 Transfer of hyl_{Efm} plasmids

The *in vitro* transfer of large-sized plasmids containing hyl_{Efm} genomic island (GI) was achieved by filter mating conjugation using seven *Enterococcus faecium* donor and seven recipient strains, four of which were *E. faecium* and three *E. faecalis*. Filter mating conjugation experiments showed different transfer rates of hyl_{Efm} plasmids and different mating rates among the mating pairs tested. Transfer of hyl_{Efm} plasmids occurred at a higher frequency when *vanA* and hyl_{Efm} were located onto the same plasmid (Table 4.1). Strain UW5905 yielded no transconjugants. S1-nuclease digestion and Southern hybridization analysis of some of the transconjugants showed sporadic changes in the plasmid size and in the presence of hyl_{Efm} or *vanA* genes in the acquired plasmids after horizontal transfer (Table 4.2, Figure 4.2 and Figure 4.1) Conjugation experiments yielded the transconjugant strain 64/3xUW3114 bearing only pLG1. Further transfer of pLG1 from this transconjugant into the recipient strain 64SS was also confirmed (Table 4.1). Transfer of hyl_{Efm} plasmid without transfer of other plasmids, suggests that hyl_{Efm} plasmids are self transferable. Later sequence analysis confirmed this observation (see below). I-*Ceu*I macrorestriction analysis of the transconjugants showed that no chromosomal integration of hyl_{Efm} had occurred (Figure 4.3 and Figure 4.4)

4.2 hyl_{Efm} Genomic environment

4.2.1 hyl_{Efm} Genomic island structure

Search of the hyl_{Efm} GI in the databases and comparison of the sequences resulted in re-annotation of the hyl_{Efm} GI into a 17,817 bp structure by joining fragments of contig 547 (5948:23 bp) (AAAK03000114.1) and contig 625 (1374:12729 bp) (AAAK03000042.1) of DO whole genome shotgun sequences (Figure 4.5). PCR reaction using primersctg625DOdelF / ctg625DOdelR and HylPAIgapF / HylPAIgapR confirmed the joint of these two contigs. The hyl_{Efm}GI structure was also found identical (by microbial BlastN) in 6 other *E. faecium* microbial genomic contigs (ACIY01000535.1, ACOB01000003.1, ACJQ01000003.1, ACAX01000152.1, ACBA-

Donor strain	Recipient strain	Selection (mg/L)	hyl_{Efm} - *vanA* co-localization	Mating rate[a]	hyl_{Efm} positive T
	E. faecium				
UW2460	64/3	VAN5-RAM30-FUS20	no	$4.75x10^{-8}$	5/20
UW5904	64/3	VAN5-RAM30-FUS20	no	$< 1.25x10^{-9}$	-
UW5905	64/3	VAN5-RAM30-FUS20	no	$< 1.25x10^{-9}$	-
UW3308	64/3	VAN5-RAM30-FUS20	no	$6.43x10^{-6}$	0/22
UW2774	64/3	VAN5-RAM30-FUS20	yes	$2.8x10^{-6}$	23/24
UW6715[b]	64/3	ERY5-FUS20-RAM30	van^s	$7.14x10^{-6}$	0/20
UW6717[b]	64/3	ERY5-FUS20-RAM30	van^s	$7.14x10^{-6}$	2/20
UW2774	BM4105RF	VAN5-RAM30-FUS20	yes	$5.54x10^{-5}$	n-t
UW2774	GE-1	VAN5-RAM30-FUS20	yes	$7.48x10^{-05}$	n-t
64/3xUW2774	64SS	VAN4-STR500-SPE300	yes	$4.4x10^{-02}$	3/4
	E. faecalis				
UW2774	OG1RF	VAN5-RAM30-FUS20	yes	$< 5,05x10^{-07}$	-
UW2774	JH2-2	VAN5-RAM30-FUS20	yes	$< 3,72x10^{-06}$	-
UW2774	UV202	VAN5-RAM30-FUS20	yes	$< 2,89x10^{-06}$	-

Table 4.1: Conjugative transfer of plasmids containing the hyl_{Efm} GI by filter mating. Notice that no transfer into *E. faecalis* could be achieved. [a]Expressed as number of transconjugants (T), VAN or ERY resistant, per recipient cell. van^s, vancomycin sensitive; FUS, fusidic acid; RAM, rifampicin VAN, vancomycin; ERY, erythromycin; STR, streptomycin, SPE, spectinomycin, n-t, not tested.

Strain	plasmids present (calculated size in Kb)				
UW2774	281 (h,v)		75		
64/3x UW2774 T-1	281 (h,v)				
T-2	226(h)	180,75 (h)	168,09 (h)		
T-3	286 (h)				
T-4	275	93,35 (v)			
T-5	310		85 (v)		
UW2460	250,13	213,57 (h)	75,87	33 (v)	9,87
64/3x UW2460 T-1			86,63		
T-2	247,75	213,71 (h)		29,1(v)	9,95
T-3	248,79			30,04 (v)	9,94
T-4		213,17 (h)		31,7 (v)	
T-5	254,78	214,68 (h)		33,89 (v)	9,89

Table 4.2: Plasmid content of selected transconjugants, calculated based on S1-nuclease PFGE analysis. Presence of hyl_{Efm} (h) and/or *vanA* (v) onto the plasmid are indicated. T, transconjugant

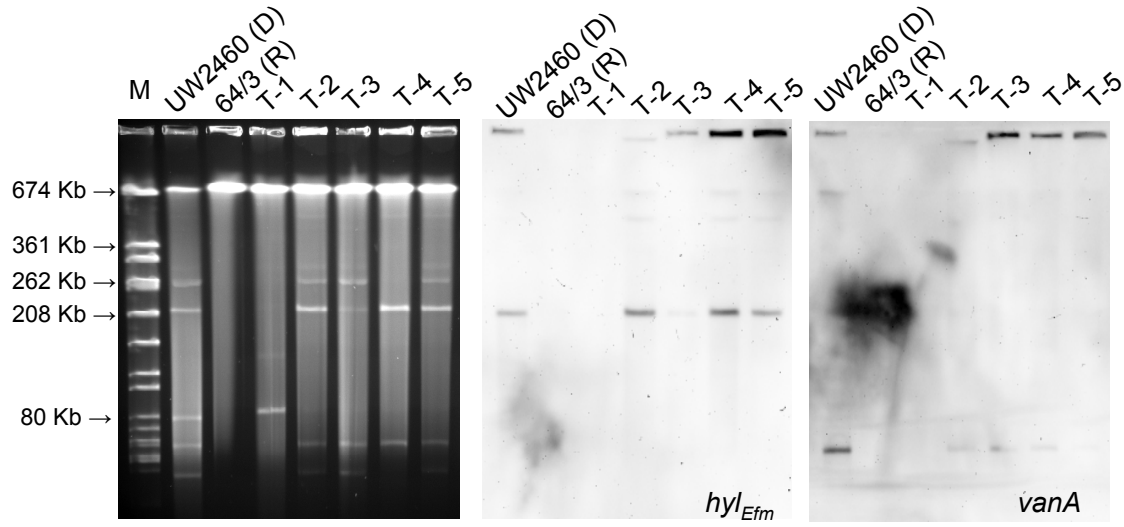


Figure 4.1: S1-nuclease analysis resolving plasmid content of donor strain UW2460, recipient strain 64/3 and its corresponding transconjugants. Notice variations in hyl_{Efm} *vanA* plasmid sizes after transfer. Southern hybridization membranes using hyl_{Efm} and *vanA* probes are also shown. T-1, T-2, T-3, T-4 and T-5 are 64/3xUW2460 transconjugants. T-1 is hyl_{Efm} negative. The uppermost bands visible are the pockets. The bands migrating at an apparent size of ca. 674 kb visible in all lanes and hybridizing to hyl_{Efm} probe correspond to undigested chromosomal and plasmid DNA.

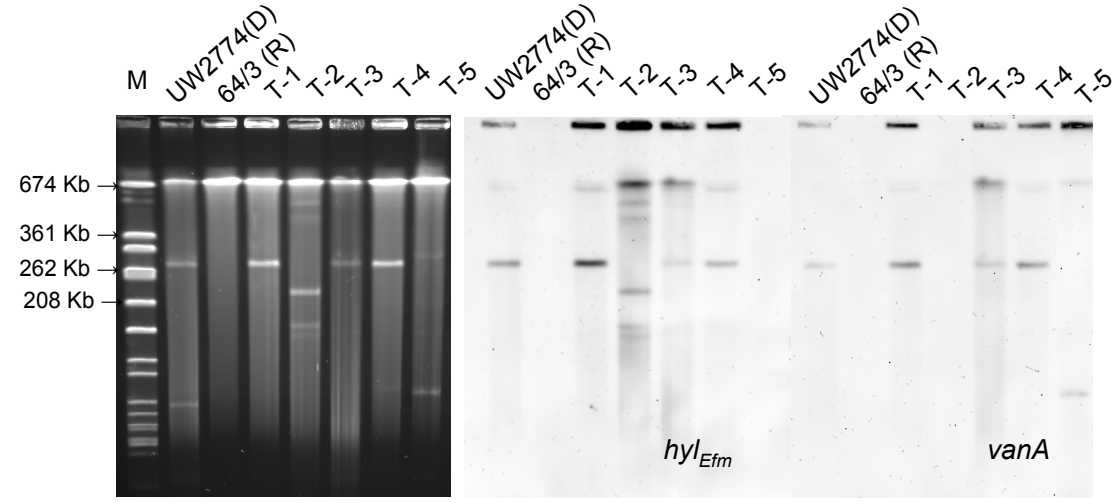


Figure 4.2: S1-nuclease analysis resolving plasmid content of donor strain UW2774, recipient strain 64/3 and its corresponding transconjugants. Notice variations in hyl_{Efm} *vanA* plasmid sizes after transfer. Southern hybridization membranes using hyl_{Efm} and *vanA* probes are also shown. 64/3xUW2774 T-1, transconjugant used for plasmid pLG1 sequencing, T-2, T-3, T-4, T-5, additional 64/3 x UW2774 transconjugants. T-5 is hyl_{Efm} negative. The uppermost bands visible are the pockets. The bands migrating at an apparent size of ca. 674 kb visible in all lanes and hybridizing to hyl_{Efm} probe correspond to undigested chromosomal and plasmid DNA.

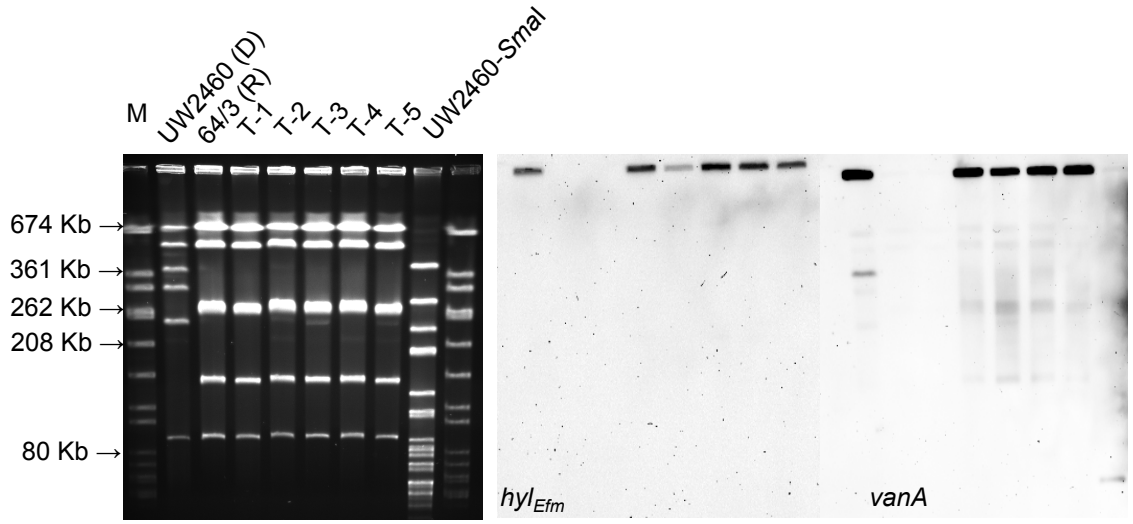


Figure 4.3: I-*Ceu*I macrorestriction analysis resolving only chromosomal bands of donor strain UW2460, recipient strain 64/3 and its corresponding transconjugants. Southern hybridization membranes using hyl_{Efm} and *vanA* probes are also shown. T-1, T-2, T-3, T-4 and T-5 are 64/3xUW2460 transconjugants. T-1 is hyl_{Efm} negative. The last lane shows the *Sma*I restriction pattern of the donor strain. The uppermost bands visible are the pockets.

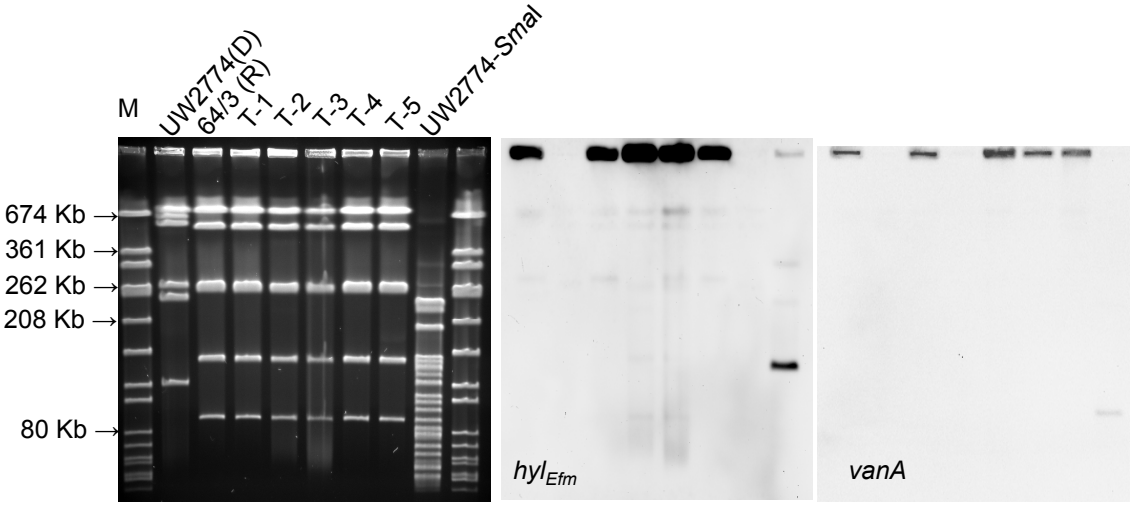


Figure 4.4: I-*Ceu*I macrorestriction analysis resolving only chromosomal bands of donor strain UW2774, recipient strain 64/3 and its corresponding transconjugants. Southern hybridization membranes using hyl_{Efm} and *vanA* probes are also shown. 64/3xUW2774 T-1, transconjugant used for plasmid pLG1 sequencing, T-2, T-3, T-4, T-5, additional 64/3 x UW2774 transconjugants. T-5 is hyl_{Efm} negative. The last lane shows the *Sma*I restriction pattern of the donor strain. The uppermost bands visible are the pockets.

01000164.1 and ACAS01000191.1). For investigation of the hyl_{Efm} GI structure, long template PCRs amplifying overlapping regions of ca. 5.5 kb (hylGI1, hylGI2 and hylGI3) were done (Figure 4.5). Later sequencing of hyl_{Efm} plasmid pLG1 allowed confirmation of the re-annotated hyl_{Efm} genomic region (HM565216) and Table 4.4). The putative hyl_{Efm} GI structure was then investigated in 39 clinical *E. faecium* isolates revealing a conserved structure. Twenty of the investigated isolates yielded no product in region hylGI3c (Table 4.3). No association could be established between the negative PCR in region 3c and a certain MLST type, the plasmid size, the presence of *vanA* or the year of isolation. Presence of an insertion into region hylGI3c was resolved in pLG1 amplifying region hylGI3c by long template PCR and sequencing using a series of primers hylPAI3 seq (see Table A. 1). The hyl_{Efm}GI has a 2,344 bp insertion downstream of hyl_{Efm} in the intergenic region between the GMP synthase (pLG-0216) and a conserved hypothetical protein (pLG-0213) putative genes (Table 4.4, Figure 4.5). The integration of IS*66* was not investigated in all strains. This insertion is an incomplete copy of IS*66* and bears two ORFs (pLG-214, IS66 Orf2 like and pLG-215, Transposase IS66) and 25 bp inverted repeats (GTAAGCGCCCCATAGAACAGTACCT) at coordinates 14851:14875 bp and 17171:17195 bp (in contig 65). The 8 bp DR at the insertion site were not present. The identified IS*66* of pLG1 contains two transposases which have a conserved Integrase core domain of the rve superfamily (cl01316). The IS*66* element of pLG1 lacked of significant similarity (blastN) to the classical IS*66*, IS*679* of *Escherichia coli* (NC_002695, NC_002142). This insertion is absent from the hyl_{Efm} GI of strain DO and from the 6 *E. faecium* genomic contigs where the hyl_{Efm} genomic region is conserved (see above).

The putative GI is flanked by two incomplete inverted copies of the *E. faecium* IS*1476* (U63997) putative transposase gene. Presence of the complete two flanking IS was not confirmed. The transposase IS*1476* gene is 1500 bp and only confirm presence of a 501 bp und 753 bp fragments up and downstream of the island could be confirmed. The regions flanking the putative GI outside of IS*1476* could not be determined neither by primer walking (Seegene, Seoul, Korea) nor by 454 sequencing and data assembly. In the DO contig 625 which was initially used for annotation of the hyl_{Efm} GI, the sequence extends downstream of the 3' flanking IS*1476*. PCR using primers Hyl PAI-4F/Hyl PAI-4R, enzymatic digestion and sequencing of the amplified products showed that this region downstream of hyl_{Efm} GI is specific for strain DO but not for the other strains tested: U0317, UW2460 and UW2774. The presence of the IS*1476* at the ends of they GI prevented the resolution of the GI flanking regions by genomic walking *Speedup* ®, since amplicons large enough to provide the sequences of the hyl_{Efm} GI flanking regions are out of the limits of detection of the method. The reannotated first two genes of the putative hyl_{Efm} GI are predicted to encode a two component system transcription regulation operon consisting of a sensor histidine kinase (pLG-0203) and a response regulator (pLG-

0204). A frame shift in strain DO contig 547 (AAAK03000114.1), absent in pLG1, caused a premature stop codon in the response regulator gene located downstream of the 5' flanking IS.

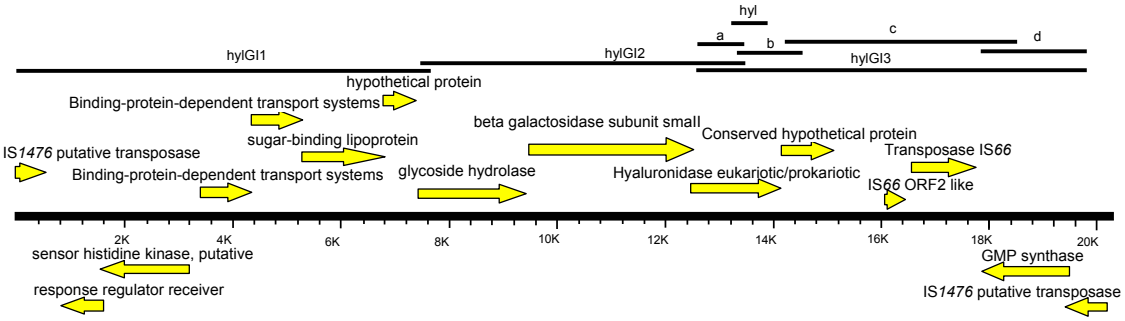


Figure 4.5: Structure of the genomic environment of hyl_{Efm} in pLG11. Solid bars show fragments amplified by PCR. hylGI1, hylGI2 and hylGI3 correspond to the regions amplified by long template PCR, (hylGI3-) a, b, c, d and hyl are regions amplified by regular PCR.

4.2.2 hyl_{Efm} Localization in *E. faecium* isolates

The hyl_{Efm} gene was localized on megaplasmids ranging from 150 kb to 350 kb in 37 of 39 clonally different *E. faecium* strains analyzed (Figure 4.6). Among the *vanA* positive strains (n=12) *vanA* and hyl_{Efm} were localized on the same plasmid in four isolates (including pLG1) while eight carry these genes on different plasmids (Figure 4.7, Table 4.3). We did not see hybridization of hyl_{Efm} to any of the plasmid bands of strains DO and UW6379 (Figure 4.6). Instead, Southern hybridization of I-*Ceu*I PFGE showed hybridization to a chromosomal band (data not shown) suggesting that in these two strains hyl_{Efm} is integrated in the chromosome.

4.3 Hyaluronidase activity

Two different methods to evaluate hyaluronidase activity were established. None of the 39 enterococcal strains tested were able to lyse hyaluronic acid despite being designated as hyl_{Efm} positive. The predicted hyl_{Efm} protein sequence was searched for protein domains and functional sites stored in Prosite database (`http://expasy.org/prosite/`) aiming to predict an alternative function, no motifs were found. Figure 4.8 shows an example of positive and negative reactions observed in both tests used.

4.4 hyl_{Efm}-megaplasmid (pLG1) sequencing

Sequencing of hyl_{Efm} *vanA* megaplasmid pLG1 lead to findings regarding the backbone and classification of this plasmid, as well as possible contribution to metabol-

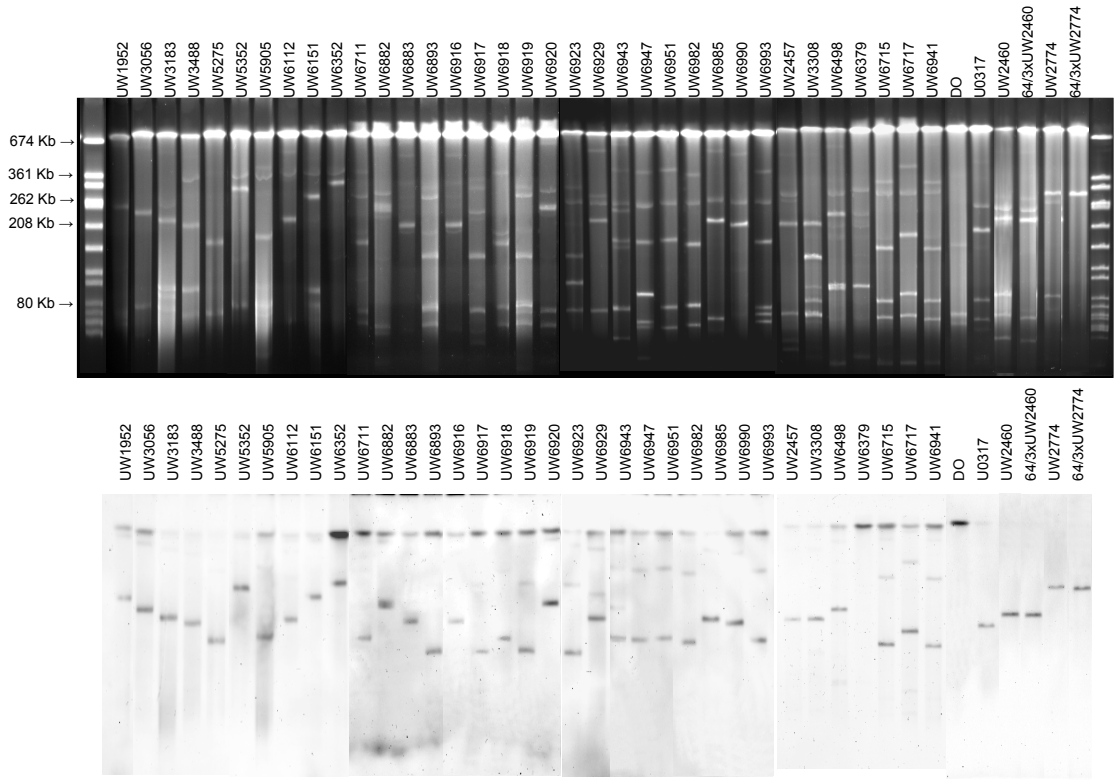


Figure 4.6: S1-nuclease PFGE of 39 hyl_{Efm} positive isolates is shown (top) and corresponding Southern hybridization membranes using hyl_{Efm} probe (bottom). Characteristics of the strains and the calculated hyl_{Efm} plasmid sizes are described in Table 4.3. Linearized plasmids are resolved in the gel on a background of degraded chromosomal DNA. The upper bands (migrating at an apparent size of ca. 674 kb) visible in all lanes and hybridizing to hyl_{Efm} probe correspond to undigested chromosomal and plasmid DNA.

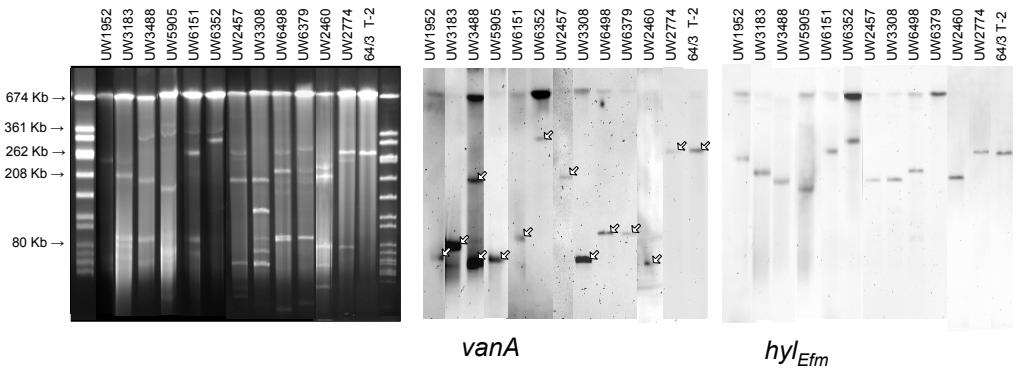
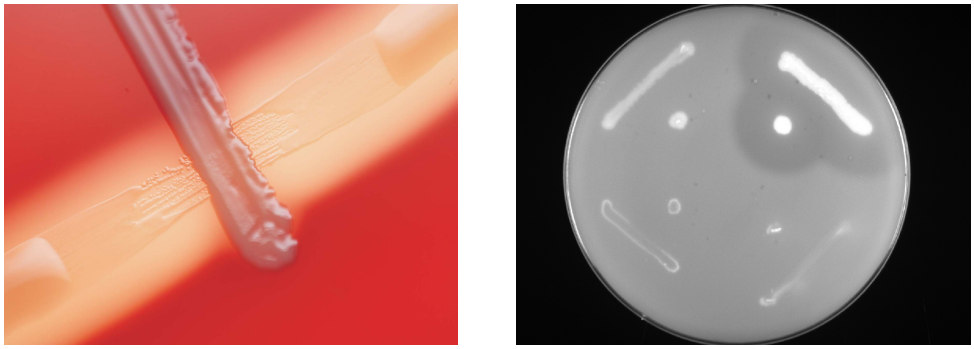


Figure 4.7: S1-nuclease PFGE of 12 strains hyl_{Efm} and *vanA* positive and the corresponding Southern hybridization membranes using hyl_{Efm} and *vanA* probes. Transconjugant strain 64/3xUW2774 (T-1) is also included. Arrows point at the hybridization signal of the *vanA* probe. Linearized plasmids are resolved in the gel on a background of degraded chromosomal DNA. Characteristics of the strains and the calculated hyl_{Efm} plasmid sizes are described in Table 4.3. The upper band (migrating at ca. 674 kb) visible in all lanes correspond to undigested chromosomal and plasmid DNA.



(a) (b)

Figure 4.8: (a) Positive hyaluronidase activity expressed by *Staphylococcus aureus* 8325-4 on *S. equi* hyaluronic acid-capsule degradation test. (b) Plate test using hyaluronic acid media, shows in the upper right quarter of the plate the positive control *S. aureus* and clockwise, *E. faecium* strains UW2460, UW2774 and 64/3xUW2774, with no hyaluronidase activity. None of 39 hyl_{Efm} PCR positive strains tested showed a hyaluronidase activity

ism, pathogenicity and resistance, of determinants that are encoded on this conjugative element. The *E. faecium* plasmid pLG1 sequences have been deposited at GenBank under accession numbers HM565168 to HM565236. The assembly statistics after 454-sequencing of hyl_{Efm} *vanA* megaplasmid pLG1 were the following:

- total of contigs: 99
- total number of aligned bases: 2,853,150 bp
- contigs lager than 500 bp: 64
- number of bases in contigs larger than 500 bp: 256.16 kb
- average contig size: 4,002 bp
- largest contig: 40,663 bp
- N50 contig size 7,757 bp

Of the 35 contigs smaller than 500 bp, 13 did not contain any predicted CDS and were excluded from the analysis. Given the calculated plasmid size of 281.02 kb and the total number of aligned bases of 2,853,150 bp, the covering was about 10.1 reads per bp. Difficulties in the assembly were due to the presence of several insertion sequences, transposons and other repetitive sequences. Southern hybridization was positive for all the 32 probes tested (including hyl_{Efm}) confirming location of the tested contigs onto the sequenced plasmid and not in the chromosome. Primers used for probe synthesis and putative function of the investigated CDSs are listed in Table A. 3. Eighteen PCR reactions were performed intending to close 17 gaps in the draft assembly. Fourteen of these PCR reactions yielded products, these were sequenced and its sequences were re-aligned with the pLG1 sequencing contigs. In this way a total of 17 gaps could be closed. This resulted in a final total of 69 sequencing contigs of pLG1. Primers used for closing gaps are listed in Table A. 4. The identified homologies of predicted open reading frames (ORF) can be grouped into: replication, mobilization/conjugation, resistance and metabolism as presented

below. An overview of the identified ORF and their predicted functions is presented in Table 4.4. Some of the ORFs putatively involved in replication, maintenance and mobilization found in pLG1 were investigated by PCR in the set of 39 hyl_{Efm} positive strains and some hyl_{Efm} negative strains that carry large sized- plasmids. The results are presented in Table 4.5.

4.4.1 Replication and mobilization

A 1,038 bp ORF was identified as a putative replicase gene (*repA*-pLG1 pLG-0001) based on similarity at the protein level to the RepA of pAD1 (gb|AAB00503.1) and is expected to encode a replication initiator protein, originally from *E. faecalis*. The overall similarity at the protein level between RepA_pAD1 and RepA_pLG1 is on average 54% (189/347 identities), but similarity differed in the N-terminal and the C-terminal regions (79% and 30% respectively). A putative *oriV* was also identified within the conserved C-terminal sequence of *repA*-pLG1 by similarity at the nucleotide level to the 170 bp *oriV* region of *repA*-pAD1. The direct and inverted repeats which are essential in RepA binding to *oriV*, as described by Francia *et al.*, were also highly conserved in *repA*-pLG1 [37]. Francia *et al.* have previously reported the conservation of Y41, D58, L90, L95 and Y116, in the N-terminal domain of 38 RepA homologues [37]. This motif was also found to be conserved in RepA_pLG1. The identified RepA_pLG1 consists of 436 amino acids (AA), remarkably larger than RepA_pAD1 (336 AA) and other annotated enterococcal RepA (326 to 346 AA). Phylogenetic analysis of repA_N homologues as described by Weaver *et al.* [185] was performed including the RepA_pLG1. This demonstrated that pLG1 bears a RepA_N family replicon and revealed that it fits squarely among the enterococcal sequences but constituting a single branch (Figure 4.9). An ORF (pLG-0236) with 100% similarity to the replication associated gene *repB* of pTEF3 (ref|ZP_00602517.1|) was also found in pLG1. This ORF has homology to proteins related to CDS1_ pRE25. No genetical link of *repB* and the identified *repA* could be established. A putative relaxase gene *rel*-pLG1 (pLG-0177) having 98% identity (525/535) to conserved hypothetical protein sequences of enterococci and 27% identity (136/487) to a putative relaxase of *Streptococcus macedonicus* (*gb*|*ABI*30222.1|) was also identified. Analysis of the translated sequence of this ORF showed that the relaxase domain was contained in the first 150 amino acids of the N-terminus. Phylogenetic analysis of relaxase genes according to Garcillán-Barcia *et al.* [40] was applied to Rel_pLG1 and it was seen that it belongs to the MobP family, MobP7 subfamily (Figure 4.10) and it clusters among lactococcal and *E. faecalis* relaxases. Further replication and segregation related genes present in pLG1 are presented in Table 4.4. The presence of the replicase gene of pLG1 was investigated for the set of 39 hyl_{Efm} positive strains by PCR and sequencing of a 135 bp fragment of the variable N-terminal region. This showed that *repA*-pLG1

Strain	Year	MLST	MLVA	*hyl*$_{Efm}$-pl.	van	vanA-pl.	hylGI[a] regions					
							1	2	3			
									a	b	c	d
DO	1991	18		C			+	+	+	+	+	+
UW1952	1998	18	1	252.11	vanA	58.59	+	+	+	+	-	+
UW2774	1999	18	281	281.02	vanA	281.02	+	+	+	+	-	+
UW2460	1999	17	1	213.57	vanA	27.81	+	+	+	+	+	+
UW2457	1999	80	1	205.00	vanA	205.00	+	+	+	+	+	+
UW3056	2000	65	5	228.20	0		+[b]	+	+	+	+	+
UW3488	2001	18	1	205.43	vanA	205.43/50.0	+	+	+	+	+	+
UW3183	2001	18	1	214.46	vanA	104.20	+[b]	+	+	+	+	+
UW3308	2001	65	-	206.05	vanA	44.02	+[b]	+	+	+	+	+
UW5905	2004	192	159	188.12	vanA	68.43	+	+	+	+	-	+
UW5352	2004	17	7	296.07	vanB		+	+	+	+	+	+
UW5275	2004	376	1	179.73	vanB		+	+	+	+	+	+
U0317	2005	78		195.06			+	+	+	+	+	+
UW6379	2005	18	1	C	vanA	101.45	+	+	+	+	-	-
UW6352	2005	18	1	316.39	vanA	316.39	+	+	+	+	+	+
UW6151	2005	17	1	262.60	vanA	98.38	+	+	+	+	+	+
UW6112	2005	378	5	216.19	0		+	+	+	+	+	+
UW6993	2006	192	159	187.53	0		+	+	+	+	-	+
UW6990	2006	18	7	215.97	0		+	+	+	+	+	+
UW6985	2006	81	7	222.85	0		+	+	+	+	-	+
UW6982	2006	192	159	184.69	0		+	+	+	+	-	+
UW6951	2006	192	159	189.90	0		+	+	+	+	-	+
UW6947	2006	17	7	187.24	0		+	+	+	+	-	+
UW6943	2006	192	159	189.27	0		+	+	+	+	-	+
UW6941	2006	192	12	163.64	0		+	+	+	+	-	+
UW6929	2006	192	159	265.66	0		+	+	+	+	-	+
UW6893	2006	192	159	169.07	0		+	+	+	+	-	+
UW6883	2006	378	new	216.94	0		+	+	+	+	-	+
UW6882	2006	117	282	255.15	0		+	+	+	+	+	+
UW6717	2006	317	-	186.93	0		+	+	+	+	-	+
UW6715	2006	192	159	165.5	0		+	+	+	+	-	+[b]
UW6711	2006	192	159	190.41	0		+	+	+	+	-	+
UW6498	2006	18	7	222.63	vanA	101.04	+	+	+	+	+	+
UW6923	2007	202	1	167.78	0		+	+	+	+	-	+
UW6920	2007	117	282	250.52	0		+	+	+	+	+	+
UW6919	2007	192	159	167.98	0		+	+	+	+	-	+
UW6918	2007	17	7	187.07	vanB		+	+	+	+	-	+
UW6917	2007	17	1	168.20	0		+	+	+	+	-	+
UW6916	2007	378	new	216.98	0		+	+	+	+	+	+

Table 4.3: *hyl*$_{Efm}$ location and *hyl*$_{Efm}$ GI structure in 39 *E. faecium hyl*$_{Efm}$ positive clinical strains. Aproximate *hyl*$_{Efm}$ and *vanA* plasmid (pl) sizes are given in kb and were calculated by S1-nuclease PFGE analysis. [a] hyl genomic island (hylGI) presence was determined by PCR (see Figure 4.5). [b] Product larger than expected. C: the results indicate chromosomal localization.

Locus Tag	Contig	Accession number	Coordenates	Product
hyl_{Efm} genomic island				
pLG1-0203	65	HM565216	347:1075	response regulator receiver [*E. faecium* 1,230,933]
pLG1-0204	65		1053:2639	sensor histidine kinase, putative [*E. faecium* U0317]
pLG1-0205	65		2836:3792	Binding-protein-dependent transport systems [*E. faecium* DO]
pLG1-0206	65		3804:4715	Binding-protein-dependent transport systems [*E. faecium* DO]
pLG1-0207	65		4739:6211	sugar-binding lipoprotein [*E. faecium* DO]
pLG1-0208	65		6262:6873	hypothetical protein EfaeDRAFT_1158 [*E. faecium* DO]
pLG1-0209	65		6852:8816	glycoside hydrolase [*E. faecium* 1,230,933]
pLG1-0210	65		9093:11924	beta galactosidase subunit small [*E. faecium* 1,230,933]
pLG1-0211	65		11921:13582	Hyaluronidase eukaryotic/prokaryotic [*E. faecium* DO]
pLG1-0212	65		13622:14554	conserved hypothetical protein [*E. faecium* DO]
pLG1-0213	65		14885:15127	hypothetical protein EfaeDRAFT_2591 [*E. faecium* DO]
pLG1-0214	65		15117:15470	IS66 Orf2 like [*E. faecium* DO]
pLG1-0215	65		15572:17119	Transposase IS66 [*E. faecium* DO]
pLG1-0216	65		17267:18832	GMP synthase [*E. faecium* U0317]
Replication and mobilization				
pLG1-0001	21	HM565183	40769:41809	replication-associated protein RepA [*E. faecium* 1,230,933]
pLG1-0015	21		3411:3896	Single-strand binding protein [*E. faecium* DO]
pLG1-0019	21		5746:5997	topoisomerase [*E. faecium* DO]
pLG1-0020	21		6023:7747	DNA topoisomerase [*E. faecium* DO]
pLG1-0054	21		39614:39946	positive regulator for conjugation [*E. faecium* DO]
pLG1-0055	1	HM565168	458:4825	type III restriction endonuclease [*E. faecium* 1,230,933]
pLG1-0056	1		5756:6199	putative DNA repair protein RadC [*E. faecalis* PC1.1]
pLG1-0059	1		7169:7792	Resolvase, N-terminal:helix-turn-helix region [*E. faecium* DO]
pLG1-0146	17	HM565181	394:1017	MobC filamentation induced by cAMP- Fic [*E. faecium* 1,231,410]
pLG1-0162	26	HM565186	236:871	Resolvase, N-terminal: helix-turn-helix region [*E. faecium* DO]
pLG1-0165	29	HM565187	1168:1782	site-specific recombinase, resolvase family [*E. faecium* E1071]
pLG1-0237	78	HM565222	1084:1875	hypothetical protein EfaeDRAFT_2626 [*E. faecium* DO]
pLG1-0277	94	HM565234	3880:4443	resolvase [*E. faecium* 1,230,933]
pLG1-0250	86	HM565227	9139:9693	resolvase [*S. pneumoniae* CGSP14]
pLG1-0177	35	HM565192	2109:3716	putative relaxase
Maintenance				
pLG1-0164	29	HM565187	14:1117	abortive infection bacteriophage resistance protein [*E. faecium* E1071]
pLG1-0004	21	HM565183	44100:44354	transcriptional regulator/antitoxin, MazE [*Mesorhizobium* sp. BNC1]
pLG1-0005	21		44355:44672	plasmid stabilization system protein [*E. faecium* 1,141,733]
pLG1-0006	21		2:145	PemI family protein [*E. faecalis* V583]
pLG1-0017	21		4814:5407	abortive infection protein AbiGI [*E. faecium* 1,230,933]
pLG1-0163	26	HM565186	895:1245	PemK family protein [*E. faecium* DO]
pLG1-0110	12	HM565176	7743:8012	Plasmid stabilization system [*E. faecium* DO]
T4SS-like components				
pLG1-0034	21	HM565183	18203:19408	(VirB1-like) peptidase M23B [*E. faecium* D344SRF]
pLG1-0035	21		19408:21438	(VirB4-like) ATPase [*E. faecium* 1,231,410]
pLG1-0040	21		25576:28188	(VirD4-like) conjugation protein [*E. faecium* 1,230,933]
pilin gene cluster				
pLG1-0044	21	HM565183	31362:33452	pilF conserved hypothetical protein
pLG1-0045	21		33449:33709	conserved hypothetical protein [*E. faecium* 1,231,501]
pLG1-0046	21		33722:34483	pilE conserved hypothetical protein
pLG1-0047	21		34499:35251	sortase family protein [*E. faecium* E1071]
pLG1-0048	21		35266:37242	PilA [*E. faecium*]
pLG1-0049	21		37295:37993	sortase [*E. faecium* 1,141,733]
Resistance	heavy metal			
pLG1-0167	32	HM565189	11:424	integral membrane protein [*E. faecium* 1,231,501]
pLG1-0168	32		771:3239	copper-translocating P-type ATPase [*E. faecium* E1071]
pLG1-0221	70	HM565218	3201:3434	putative copper chaperone [*E. faecium* E1071]

pLG1-0222	70		3587:5536	cadmium-translocating P-type ATPase [*E. faecium* E1071]
pLG1-0259	91	HM565231	827:1282	copper transport repressor, CopY/TcrY family [*E. faecium* E1071]
pLG1-0260	91		1295:3760	TcrA [*E. faecium*]
pLG1-0261	91		3845:4051	TcrZ [*E. faecium*]
pLG1-0262	91		4190:6352	TcrB [*E. faecium*]
pLG1-0263	92	HM565232	112:1077	CtpA [*Listeria monocytogenes*]
pLG1-0090	6	HM565172	990:1319	cadmium efflux system accessory protein [*E. faecium* 1,230,933]
	antibiotic			
pLG1-0091	6	HM565172	1606:2091	teicoplanin resistance protein [*E. faecium*]
pLG1-0092	6		2244:3161	VanY protein [*E. faecium*]
pLG1-0093	6		3338:4633	transposase [*E. gallinarum* EG2]
pLG1-0094	6		5106:5714	D-ala-D-ala dipeptidase [*E. faecium* 1,230,933]
pLG1-0095	6		5720:6799	vancomycin/teicoplanin A-type resistance protein VanA [*S. aureus*]
pLG1-0096	6		6744:7724	vancomycin resistance protein VanH [*S. aureus*]
pLG1-0097	6		7927:9081	sensor histidine kinase VanS [*E. faecium* E1071]
pLG1-0061	2	HM565169	605:1342	ErmB [*Lactobacillus plantarum*]
	Carbohydrate transport and metabolism			
pLG1-0063	3	HM565170	3013:4488	Sulfatase [*E. faecium* DO]
pLG1-0064	3		4789:5610	PTS system mannose/fructose/sorbose family IID [*E. faecium* DO]
pLG1-0067	3		7423:7902	PTS system sorbose subfamily IIB component [*E. faecium* DO]
pLG1-0068	3		7906:9684	Beta-galactosidase [*E. faecium* DO]
pLG1-0070	3		10565:11701	Radical SAM [*E. faecium* DO]
pLG1-0080	4	HM565171	4564:5424	oxidoreductase domain protein [*E. faecalis* TX0104]
pLG1-0081	4		5436:6263	3-methyl-2-oxobutanoate hydroxymethyltransferase [*E. faecalis* V583]
pLG1-0082	4		6278:7126	Pantoate-beta-alanine ligase [*E. faecium* DO]
pLG1-0083	4		7136:7531	Aspartate decarboxylase [*E. faecium* DO]
pLG1-0087	4		11004:11654	ABC transporter related protein [*E. faecium* DO]
pLG10101	8	HM565173	123:1373	PTS lactose/cellobiose IIC component [*E. faecium* DO]
pLG1-0105	12	HM565176	1641:2942	PTS lactose/cellobiose IIC component [*E. faecium* DO]
pLG1-0108	12		5395:6087	sugar isomerase [*E. faecium* 1,141,733]
pLG1-0119	12		15704:16579	Metallophosphoesterase [*E. faecium* DO]
pLG1-0120	12		16751:17698	Aldo/keto reductase [*E. faecium* DO]
pLG1-0122	12		18485:19738	extracellular solute-binding protein, family 1 [*E. faecium* DO]
pLG1-0123	12		19821:20744	Binding-protein-dependent transport systems [*E. faecium* DO]
pLG1-0126	12		22897:24585	Phosphoenolpyruvate-dependent sugar phosphotransferase system, EIIA 2 [*E. faecium* DO]
pLG1-0127	12		24740:25885	Phosphoenolpyruvate-dependent sugar phosphotransferase system, EIIA 2 [*E. faecium* DO]
pLG1-0128	12		25990:26283	Phosphotransferase system PTS, lactose/cellobiose-specific IIA subunit [*E. faecium* DO]
pLG1-0129	12		26285:26596	Phosphotransferase system, EIIC [*E. faecium* DO]
pLG1-0132	12		28886:29839	Alpha-mannosidase [*E. faecium* DO]
pLG1-0133	12		32931:34307	Bleomycin hydrolase [*E. faecium* DO]
pLG1-0134	12		34382:35200	Myo-inositol catabolism IolB [*E. faecium* DO]
pLG1-0135	12		35250:36227	Carbohydrate kinase, PfkB [*E. faecium* DO]
pLG1-0136	12		36178:38136	Pyruvate decarboxylase [*E. faecium* DO]
pLG1-0137	12		38158:39312	Iron-containing alcohol dehydrogenase [*E. faecium* DO]
pLG1-0138	12		39335:39766	GCN5-related N-acetyltransferase [*E. faecium* DO]
pLG1-0149	17	HM565181	2754:3806	membrane protein, putative [*E. faecium* DO]
pLG1-0150	17		3819:4757	Ketopantoate reductase ApbA/PanE [*E. faecium* DO]
pLG1-0151	18	HM565182	85:561	Sugar-specific permease, EIIA 1 domain [*E. faecium* DO]
pLG1-0152	18		574:2076	PTS, EIIB: EIIC [*E. faecium* DO]
pLG1-0153	18		2090:2779	N-acylglucosamine-6-phosphate 2-epimerase [*E. faecium* 1,231,502]
pLG1-0154	18		2939:3757	Helix-turn-helix protein RpiR:Sugar isomerase (SIS) [*E. faecium* DO]
pLG1-0157	18		4710:5519	ROK [*E. faecium* DO]
pLG1-0187	49	HM565202	1:174	hydrolase, HAD superfamily [*E. faecium* E1071]
pLG1-0193	55	HM565208	1:177	binding-protein-dependent transport system [*E. faecium* 1,231,502]

pLG1-0199	63	HM565214	21:248	glucose-6-phosphate isomerase [*E. faecium* TX1330]
pLG1-0200	64	HM565215	3:236	NAD-dependent epimerase/dehydratase [*E. faecium* E1071]
pLG1-0225	73	HM565219	2569:2796	ABC transporter, ATP-binding protein [*E. faecalis* V583]
pLG1-0253	87	HM565228	2860:4029	Sugar isomerase (SIS) [*E. faecium* DO]
pLG1-0254	87		4045:5040	DeoC/LacD family aldolase [*E. faecium* 1,230,933]
	phage related			
pLG1-0181	42	HM565196	87:248	head-tail joining protein [E. phage EFAP-1]
pLG1-0184	45	HM565199	2:331	bacteriophage terminase large subunit [Lactobacillus phage phiPYB5]
pLG1-0186	47	HM565201	84:194	prophage pi3 protein 45 [*E. faecium* 1,230,933]
pLG1-0142	13	HM565177	322:939	phage integrase [*E. faecium* 1,231,502]
pLG1-0062	3	HM565170	55:1149	Phage integrase:Phage integrase, N-terminal SAM-like [*E. faecium* DO]
pLG1-0164	29	HM565187	14:1117	abortive infection bacteriophage resistance protein [*E. faecium* E1071]

Table 4.4: Annotation of some of the identified pLG1 ORFs. Contig where the ORF is found and exact location are given. repA-pLG1 was arbitrarily taken as pLG-001. All contig sequences are available under accession numbers HM565168 to HM565236

is present in all hyl_{Efm} positive strains investigated, with slight differences in the nucleotide sequences (mainly 2 non-synonymous nucleotide substitutions) (Table 4.5). Three hyl_{Efm} negative strains carrying large sized plasmids were also positive. Analysis of the presence of *res*-pLG1, *mobC*-pLG1 and *rel*-pLG1 showed variable presence along the hyl_{Efm} positive strains.

4.4.2 Pili and conjugation

A copy of the entire pilin gene cluster (PGC) -1 (EU909697) is present in pLG1 (99% identity on the DNA level) (from pLG-0044 to pLG-0049). PGC-1 contains the genes encoding the two minor pilin proteins PilF (pLG-0044) and PilE (pLG-0046), a gene of unknown function (pLG-0045), a class C (pLG-0047) and class A (pLG-0049) sortase genes and the major pilin subunit pilA (pLG-0048). All the conserved motifs of the described pilin subunits [59] were found intact in pLG1. The consensus pilin motif with a conserved HLYPK motif, is present in pLG1 *pilA*; the sequence YSLEETKAPENY resembling the consensus Ebox motif was also found intact in *pilA*-pLG1 as well as the N-terminal signal sequence; finally, the VPMTG sortase substrate motif was also found. Transmission immuno-electron microscopy (TEM) imaging using anti-PilA immunogold particles, showed that the donor strain UW2774 expressed PilA pili (Figure 4.11). This phenotype could not be confirmed in any of the transconjugants that acquired pLG1 due to the low adherence of the strains to the carbon coated copper grids used to perform the TEM.

In pLG1 a set of genes are arranged in an operon like way are co-located upstream the previously described pilin gene cluster resembling a complete set of transfer genes related to Type IV secretion system (T4SS) (Table 4.4). In pLG1 the following genes related to T4SS were found:

- a VirD4 homolog conjugation protein (coupling protein with conserved WalkerA and WalkerB NTP binding motifs)

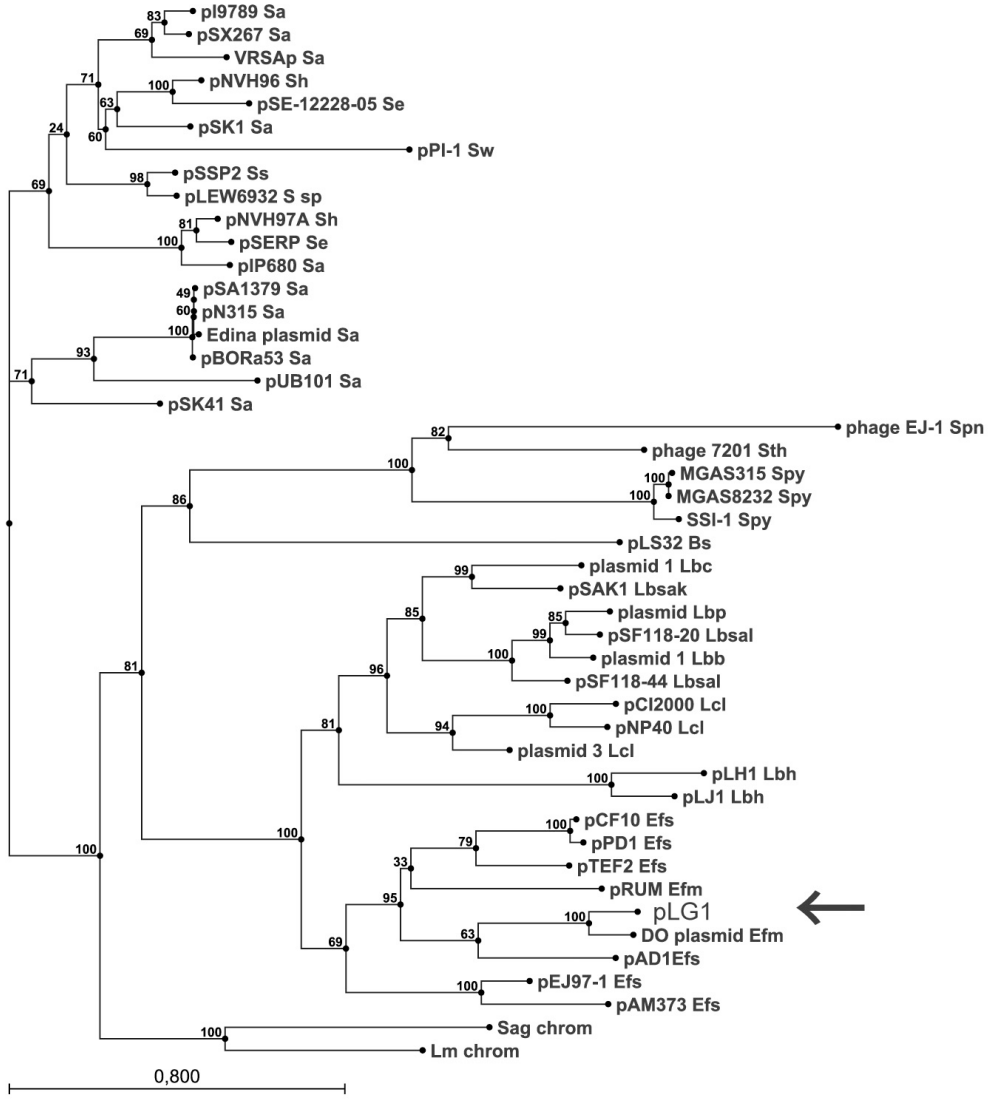


Figure 4.9: Phylogenetic alignment of Gram-positive plasmid RepA protein sequences including RepA-pLG1. The arrow points at RepA-pLG1.

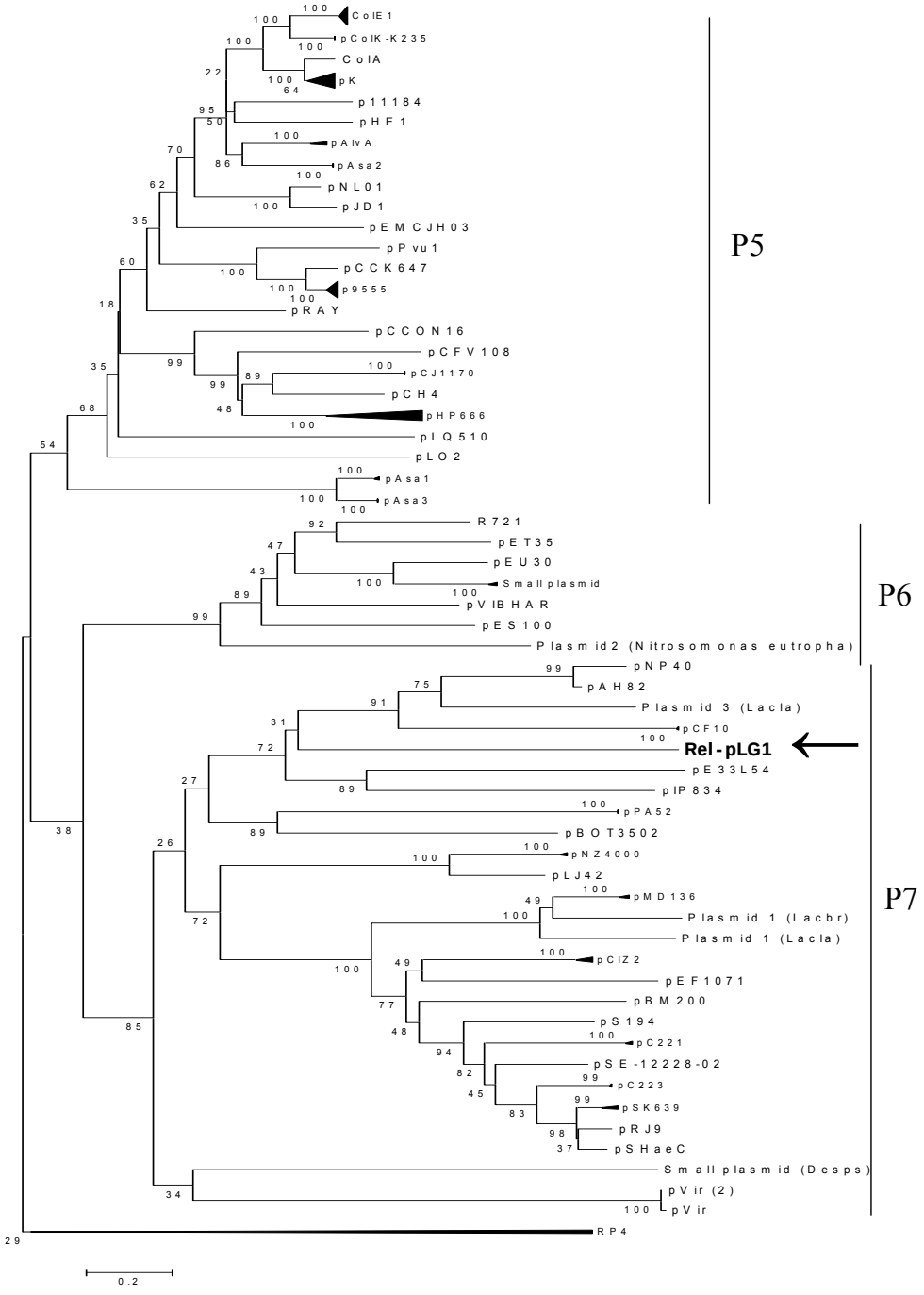


Figure 4.10: Phylogenetic alignment of MobP-family relaxase protein sequences including Rel-pLG1. The arrow points at Rel-pLG1.

- a VirB4-like ATPase (putatively coding for a 77,472.6 Da protein matching to the usual size of these ATPases (larger than 70-80 kDa, being the largest element of the putative T4SS clusters)
- a peptidase (lysozyme)
- the entire pilin gene cluster PGC-1.

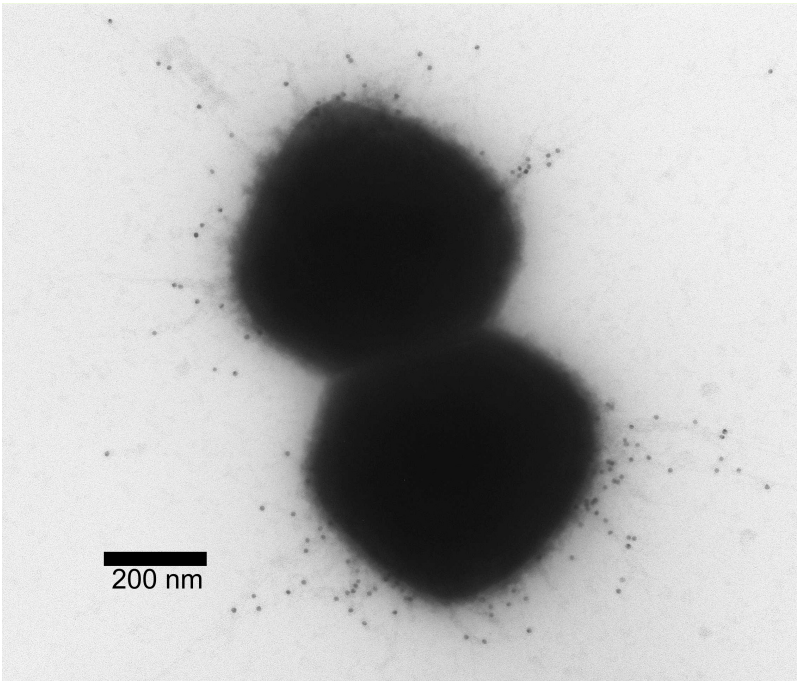


Figure 4.11: *pilA* pilin expression in strain UW2774 shown by transmission immunoelectron microscopy of cells negatively stained and labeled with immunogold (15 µm) using anti-PilA antiserum

4.4.3 Maintenance

The plasmid pLG1 has several putative coding sequences that potentially contribute to its segregational stability as listed in Table 4.4). Among them pLG-0004 and pLG-0005 seem to constitute a complete plasmid stabilization module. The predicted proteins have small sizes (9.3 kDa and 12.2 kDa respectively) and show an overlapping organization. Despite homology (37% identity) of pLG-002 to a MazE component, search of the complete *mazEF* region of *E. coli* among pLG1 sequences did not retrieve matches that indicated the presence of the entire *mazEF* system. Functionality tests of these and other putative plasmid maintenance systems should be performed in order to determine the genes that are responsible for the stability of hyl_{Efm} and *van* plasmids among the enterococcal population.

4.4.4 Resistance

Several genes encoding heavy metal resistance were found on pLG1 (Table 4.4) including a complete *tcrYAZB* operon encoding for copper resistance. A complete

vanA operon was identified in pLG1 (Table 4.4). An insertion between *vanY* and *vanX* was detected. Presence of a transposase (IS*204*/IS*1001*/IS*1096*/IS*1165*) was partially resolved, with similarity at the nucleotide level to IS*Efa5*. This *vanA* cluster might then constitute one subtype of Tn*1546*-like elements. *erm*(B) which encodes resistance to macrolides, lincosamides and streptogramin B antibiotics was also identified on pLG1. The acquired antibiotic resistance determinants observed by plasmid sequencing was congruent with the observed antibiotic resistance of the transconjugants, which acquired besides the selection marker vancomycin resistance, also macrolide resistance conferred by *erm*(B).

4.4.5 Metabolism

As previously mentioned the genes neighboring the putative hyl_{Efm} gene seem to be involved in carbohydrate metabolism. In the same way several of the identified ORFs in pLG1 are putative metabolic genes that might be involved in uptake and utilization of different carbohydrates. Several of the identified ORFs show homology to phosphotransferase systems (PTS). Neighboring all the identified PTS components identified putative CDSs related to sugar carbon metabolism were also found, including those of the hyl_{Efm} GI (Table 4.4).

4.5 Transfer of the *E. faecalis* PAI

Transfer of the *esp* gene by selecting for the transfer of antibiotic resistance determinants was pursued by filter mating. The *esp* gene of *E. faecalis* was transfered from donor strain UW3114 into *E. faecalis* and *E. faecium* recipients along with the transfer of plasmid-encoded erythromycin resistance. One conjugation experiment using *E. faecium* strain 64/3 as recipient yielded one *esp* positive transconjugant (strain 64/3xUW3114 T-10) among 14 transconjugants tested. In contrast, five conjugation experiments using *E. faecalis* strain OG1RF as recipient, resulted in no *esp* positive transconjugants among 600 transconjugants screened. The stimulating influence of SOS response in the transfer of genetic elements was assessed by exposing the donor strain UW3114 to subinhibitory concentrations of ampicillin and ciprofloxacin. When ciprofloxacin challenge was used, one transconjugant carrying *esp* was identified (strain OG1RFxUW3114 T-12) among 100 transconjugants screened (Table 4.6).

PCR analysis of overlapping regions covering the entire PAI (153.57 kb as in reference strain MMH594) demonstrated that the transferred PAI of donor strain UW3114 differed from the PAI in the reference strain MMH594. However, no difference was observed between the donor and the transconjugants, indicating that the PAI did not undergo any changes during horizontal transfer (Figure 4.12, Table 4.7). Approximately 90.6 kb of the reference PAI -MMH594 were present in the

Strain	repA-pLG1	res-pLG1	mobC-pLG1	rel-pLG1
DO	+	+	-	-
UW1952	+	+	-	-
UW2774	+	+	+	+
UW2460	+	-	+	-
UW2457	+	-	+	-
UW3056	+	-	+	-
UW3488	+	-	-	-
UW3183	+	-	-	-
UW3308	+	+	+	+
UW5905	+	+	-	-
UW5352	+	-	+	-
UW5275	+	-	-	-
U0317	+	-	-	-
UW6379	+	-	-	-
UW6352	+	-	+	-
UW6151	+	-	+	-
UW6112	+	-	+	-
UW6993	+	+	- [a]	-
UW6990	+	-	+	-
UW6985	+	+	- [a]	-
UW6982	+	+	- [a]	-
UW6951	+	+	- [a]	-
UW6947	+	+	- [a]	-
UW6943	+	+	-	-
UW6941	+	+	-	-
UW6929	+	+	-	-
UW6893	+	+	-	-
UW6883	+	-	-	-
UW6882	+	-	-	-
UW6717	+	+	-	-
UW6715	+	+	-	-
UW6711	+	+	-	-
UW6498	+	-	-	-
UW6923	+	+	- [a]	-
UW6920	+	-	- [a]	-
UW6919	+	+	-	-
UW6918	+	+	-	-
UW6917	+	-	-	-
UW6916	+	-	-	-
hyl_{Efm} negative strains:				
UW6470	+	-	-	-
UW5868	+	-	-	-
UW6463	+	-	-	-
64/3	-	-	-	-

Table 4.5: Replicase, resolvase, mobC and relaxase of pLF1 investigated in 39 *E. faecium* hyl_{Efm} positive clinical strains. Aproximate hyl_{Efm} and *vanA* plasmid (pl) sizes, given in kb are also shown. [a] faint PCR amplicon with the expected size was observed. C: chromosomal localization.

Donor strain	Recipient strain	Selection (mg/L)	Mating rate[a]	*esp* positive transconjugants
E. faecalis	***E. faecalis***			
UW3114	OG1-RF	OTE5-RAM30-FUS20	$5.8x10^{-6}$	0/200
UW3114	OG1-RF	ERY10-RAM30-FUS20	$4.7x10^{-6}$	0/300
UW3114	OG1-RF	STR500-RAM30-FUS20	$1.4x10^{-5}$	0/100
UW3114[a]	OG1-RF	ERY10-RAM30-FUS20	$1,3x10^{-7}$	1/100
UW3114[b]	OG1-RF	ERY10-RAM30-FUS20	$1,3x10^{-8}$	0/100
E. faecalis	***E. faecium***			
UW3114	64/3	ERY10-RAM30	$2.5x10^{-8}$	1/14

Table 4.6: Conjugative transfer of *esp* by filter mating. Notice that the *esp* gene of *E. faecalis* was transferred into *E. faecalis* and *E. faecium*. Donors grown in subinhibitory concentrations of [a] ampicillin or [b] ciprofloxacin to induce stress and SOS response.

PAI of UW3114 including the aggregation substance gene, the complete cytolysin operon and the entire *esp* gene. Long PCR bridging the gaps of the putative absent regions confirmed deletions located in regions: 2b-2c, 4b-5a and 9. No regular or long PCR amplification was possible that closed the gaps in regions 6a-6b and 7b-8b, suggesting that elements too large to be amplified were present (Figure 4.12, Table 4.7). The size of the transferred PAI element and its chromosomal integration were determined by PFGE and Southern hybridization. *Sma*I-PFGE analysis revealed that a single restriction fragment in each transconjugant strain was enlarged and hybridized to the *esp* probe (Figure 4.13). The fragment size shift appeared to be ca. 200 kb (calculated sizes were 206 kb in *E. faecalis* and 193 kb in *E. faecium*) and corresponded to the total size of the PAI transferred element. Precise integration (presence of 3' and 5' ends) of the whole PAI was confirmed (see below Sect. 4.5.1). Given that only 90.6 kb of the reference PAI were confirmed to be present and that additional DNA seemed to be integrated within certain regions (see above), it is likely that additional ca. 119.4 kb DNA were inserted within the PAI (Figure 4.12). S1-nuclease analysis demonstrated that the *E. faecalis* and *E. faecium* transconjugants acquired a ca. 66 kb plasmid (pLG2) (Figure 4.13), which did not hybridize to the *esp* probe, but to the selection marker *erm*(B). I-*Ceu*I macrorestriction analysis confirmed localization of *esp* on a chromosomal band in both transconjugant strains (Figure 4.14).

4.5.1 Integration site

The integration site of the *E. faecalis* PAI in the chromosome of *E. faecalis* strain OG1RF ($NZ\_ABPI01000001.1$) is at coordinates 374617:374626. This integration site was found by comparing the OG1RF genome to the chromosomal region spanning the *E. faecalis* PAI in the genome of *E. faecalis* strain V583 ($gb|AE016830.1|$) (PAI coordinates, 445126:583433). PCR amplification of the integration site of the PAI prior to its insertion confirmed that it is intact in the recipient strain OG1RF

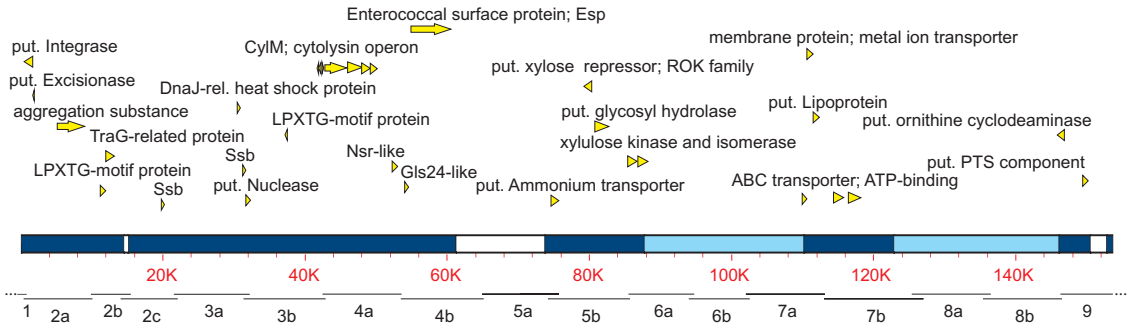


Figure 4.12: The *E. faecalis* PAI of strain UW3114 that was horizontally transfered. The structure was investigated based on the *E. faecalis* PAI of strain MMH594 (*gb*|*AF*454824.1|), by long template PCR, regular PCR, sequencing and Southern hybridizations. The long template PCR regions are indicated by horizontal lines (1 to 9) (Table A. 2). Regions present are shown in dark blue and sum up to ca. 90.6 kb. White regions are absent. Light blue regions are absent and hold additional insertions. Only representative ORFs from the regions present are shown.

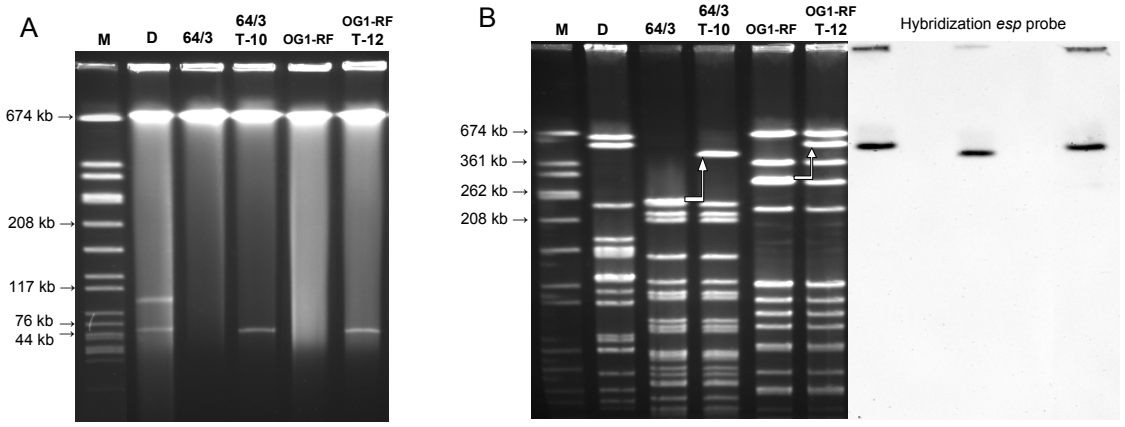


Figure 4.13: A. S1-nuclease analysis showing transfer of a ca. 66 kb conjugative plasmid into both transconjugants. None of the plasmid bands hybridized to an *esp* probe (not shown). B. *Sma*I restriction analysis and corresponding Southern hybridization using an *esp* probe. M, Marker *Staphylococcus aureus* 8325 *Sma*I-digested; D, Donor UW3114; 64/3, *E. faecium* recipient; 64/3 T-10, *E. faecium* transconjugant 64/3xUW3114 T-10; OG1-RF, *E. faecalis* recipient; OG1-RF T-12, *E. faecalis* transconjugant OG1RFxUW3114 T-12. White arrows indicate the fragment size shift due to PAI acquisition in *E. faecalis* (ca. 206 kb larger) and *E. faecium* (ca. 193 kb larger).

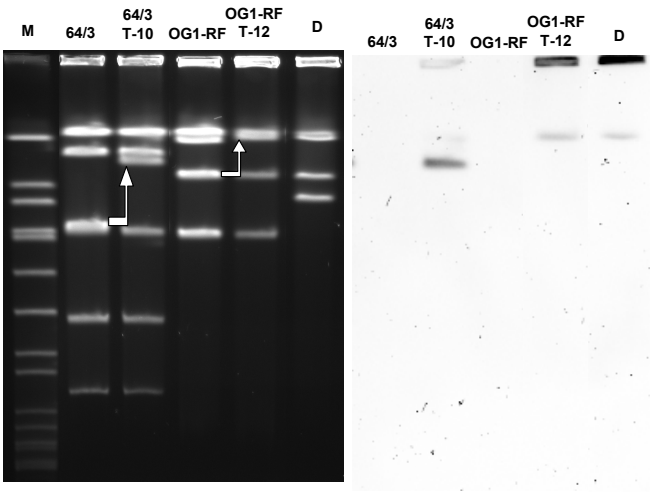


Figure 4.14: CeuI restriction analysis of the mating strains after *E. faecalis* PAI transfer. M, Marker *Staphylococcus aureus* 8325 *Sma*I-digested; D, Donor UW3114; 64/3, *E. faecium* recipient; 64/3 T-10, *E. faecium* transconjugant 64/3xUW3114 T-10; OG1-RF, *E. faecalis* recipient; OG1-RF T-12, *E. faecalis* transconjugant OG1RFxUW3114 T-12. White arrows indicate the fragment size shift due to PAI acquisition in the transconjugants.

and occupied by the PAI in the transconjugant OG1RFxUW3114 T-12. The region where the PAI integrated into the *E. faecium* transconjugant 64/3xUW3114 T-10 genome was identified to be a tRNA*lys* gene, which is located in the *E. faecium* U0317 contig00182 (*gb*|*ABSW*01000168.1|) at coordinates 47388:47460 (Figure 4.15). PCR amplification confirmed that the identified integration site tRNA*lys* was intact in the recipient strain 64/3 and occupied by the *E. faecalis* PAI in the transconjugant 64/3xUW3114 T-1(Figure 3.2). BLAST comparisons of the *E. faecalis* and the *E. faecium* regions where the *E. faecalis* PAI integrated revealed that they share 85% similarity at the nucleotide level along a 94 and 93 bp region respectively. A 10 bp (AATTCTCAGT) sequence (resembling *attB*) was found to be present at the PAI integration site of the *E. faecium* and *E. faecalis* recipient strains. PCR and sequencing of the flanking ends of the PAI and chromosomal regions spanning it demonstrated the exact integration of the PAI and confirmed the presence of the two 10 bp direct repeats (DR) (resembling *attl* and *attR*).

The region where the esp_{Efm} PAI is located in *E. faecium* (*E. faecium* U0317 Contig 00248 (*gb*|*ABSW*01000226.1|) [179]) was amplified by PCR and confirmed to be free in the recipient (64/3) and transconjugant (64/3xUW3114 T-10) strains, indicating that neither the *E. faecium* nor the *E. faecalis* PAI are integrated in this chromosomal region in the *E. faecium* donor and transconjugant strains.

4.5.2 PAI excision and circularization

Presence of a circular intermediate formed from the precisely excised PAI element could be detected by nested PCR in the donor strain UW3114. Sequencing of these amplicons confirmed that the PAI can precisely excise at its flanking ends and that these ends join together, resulting in circularization of the PAI element (See Fig.

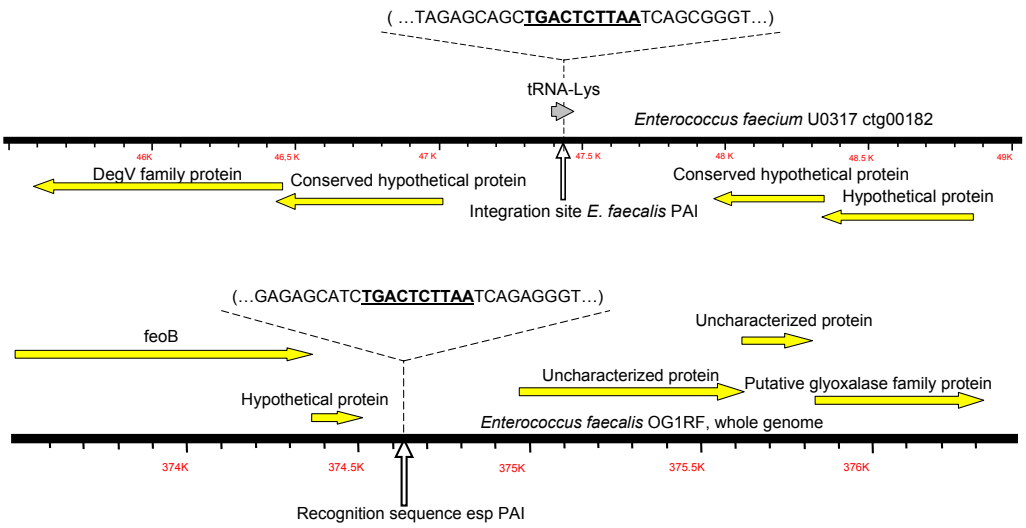


Figure 4.15: Chromosomal region where the *E. faecalis* PAI integrated in *E. faecium* as in strain U0317 $gb|ABSW01000168.1|$ (top) and *E. faecalis* OG1RF $ref|NZ_ABPI00000000|$ (bottom). The vertical white arrow points at the integration site of the *E. faecalis* PAI. The detailed nucleotide sequence shows the 10 bp region of recognition and integration of the PAI as underlined bold text.

Long PCR-region	MMH594	UW3114	64/3 T-10	OG1RF T-12	Regular PCR - ORF	MMH594	UW3114	64/3 T-10	OG1RF T-12
1	+	+	-	+	EF0001	+	+	+	+
2a	+	+	+	+	EF0005	+	+	+	+
2b [d]	+	-	-	-	EF0009	+	+	+	+
					EF0011	+	+	+	+
					EF0012	+	-	-	-
2c	+	+ *	+ *	+ *	EF0013	+	+	+	+
					EF0014	+	+	+	+
					EF0015	+	+	+	+
					EF0016	+	+	+	+
					EF0017	+	+	+	+
					EF0019	+	+	+	+
					EF0020	+	+	+	+
					EF0021	+	+	+	+
3a	+	+	+	+					
3b	+	+	+	+					
4a	+	+	+	+	EF0046	+	+	+	+
4b [d]	+	-	+ *	+ *	EF0055	+	+	+	+
					EF0056	+	+	+	+
					EF0057	+	-	-	-
					EF0058	+	-	-	-
					EF0059	+	-	-	-
					EF0060	+	-	-	-
5a	+	-	-	-	EF0061	+	-	-	-
					EF0062	+	-	-	-
					EF0065	+	-	-	-
					EF0066	+	-	-	-
					EF0067	+	-	-	-
					EF0068	+	-	-	-
					EF0069	+	-	-	-
					EF0070	+	-	-	-
					EF0072	+	+	+	+
					EF0073	+	+	+	+
5b	+	+	+	+	EF0074	+	+	+	+
					EF0076	+	+	+	+
					EF0077	+	+	+	+
					EF0078	+	+	+	+
					EF0079	+	+	+	+
					EF0080	+	+	+	+
					EF0081	+	+	+	+
					EF0082	+	+	+	+
6a [i]	+	-	-	-	EF0083	+	+	+	+
					EF0084	+	-	-	-
6b	+	-	-	-	EF0087	+	-	-	-
					EF0089	+	-	-	-
					EF0091	+	-	-	-
7a	+	-	-	-	EF0092	+	-		-
					EF0093	+	+	+	+
					EF0094	+	+	+	+
					EF0095	+	+	+	+
7b	+	+ *	+ *	+ *	EF0101	+	+	+	+
					EF0102	+	+	+	+
					EF0108	+	+	+	+
8a [i]	+	-	-	-	EF0109	+	-	-	-
					EF0111	+	-	-	-
					EF0115	+	-	-	-
8b	+	-	-	-	EF0117	+	-	-	-
					EF0119	+	-	-	-
					EF0122	+	-	-	-
					EF0123	+	-	-	-
9 [d]	+	+*	-	+*	EF0124	+	+	+	+
					EF0125	+	+	+	+
					EF0126	+	+	+	+
					EF0126	+	+	+	+
					EF0128	+	+	+	+
					EF0128-129	+	-	-	-
					EF0129	+	-	-	-
					TSP2 3′-9PAIR	+	+	-	+

Table 4.7: Results of Long template PCRs and regular PCRs screening for presence of the entire *E. faecalis* PAI (as in reference strain MMH594 [141]). PAI overlapping regions are described in A. 2 and Figure 4.12. Recipient strains were negative for all PCRs. * A product of unexpected size was amplified. [i], an insertion within this region(s) is suspected. [d], a deletion within this region(s) was confirmed.

3.2). The precisely excised circular intermediate carried the specific 10 bp sequence (AATTCTCAGT) resembling *attP*. Accordingly, excision of the PAI from the chromosome could be confirmed by amplification of the free chromosomal region that contained the PAI in the *E. faecalis* donor strain UW3114. Excision from the chro-

mosome could also be detected only after two rounds of nested PCR. In addition to precise excision and circularization of the PAI (only detected in donor UW3114), imprecise PAI excision and circularization was also observed. PCR and sequencing demonstrated that different fragments of the PAI can remain in the chromosome after excision, or chromosomal fragments can be excised together with the PAI. The following two scenarios of imprecise excision were the most frequently observed (as they were more frequently amplified in different PCR attemps): (a) the fragment 1:223 bp of PAI remains in the chromosome after PAI excision and (b) the fragment 1:1,704 bp of PAI remains in the chromosome after PAI excision (Figure 3.2). Case (a) was detected in donor strain UW3114, while case (b) was seen in reference strain MMH594, donor strain UW3114 and transconjugants 64/3xUW3114 T-10 and OG1RFxUW3114 T-12. An internal region of the PAI (coordinates 1704:1764-1784 bp) shares similarity to the chromosomal region flanking the PAI downstream, in strains MMH594 and UW3114 (50/61 identities (81%)) and OG1RF (identities=68/82, 82%). In *E. faecium* strain 64/3 this homology extends along a larger region (identities=316/393, 80%). These similarities between internal regions of the PAI and the chromosomal region flanking it downstream could trigger the imprecise excision of the PAI.

4.6 Phenotypic changes after PAI acquisition

PAI acquisition altered the phenotype of the *E. faecium* and *E. faecalis* strains. Presence of Esp on the cell surface, biofilm formation, cytolytic activity, adherence to epithelial cells and growth *in vitro* were assessed. Pathogenicity and in vivo survival of the *E. faecalis* strains were compared using mouse bacteraemia and peritonitis models.

4.6.1 Cell surface *esp* expression

Transmission immunoelectron microscopy displayed the Esp protein associated with the cell wall of the donor and transconjugant strains but not on the surfaces of the recipients (Figure 4.17). FACS analysis revealed a high level of expression of *esp* in the donor strain UW3114 as well as in the *E. faecalis* transconjugant strain OG1RFxUW3114 T-12 as compared to the reference strain MMH594. *E. faecium* transconjugant 64/3xUW3114 T-10 showed a lower *esp* expression than the *E. faecalis* strains (Figure 4.16). In all strains less Esp was expressed at lower levels at 21℃ in comparison to 37℃ (Figure 4.16).

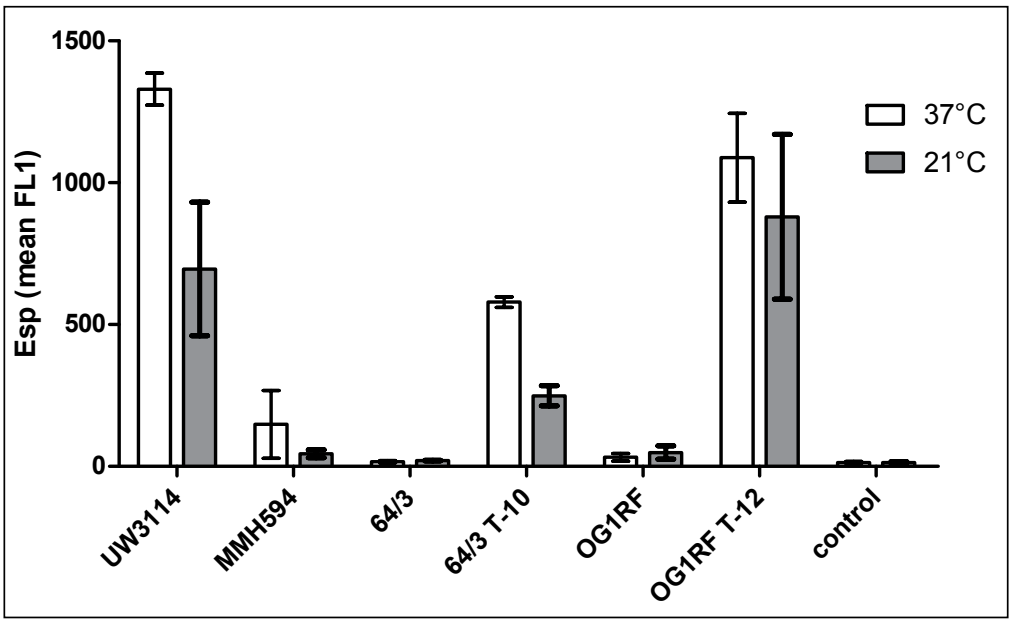


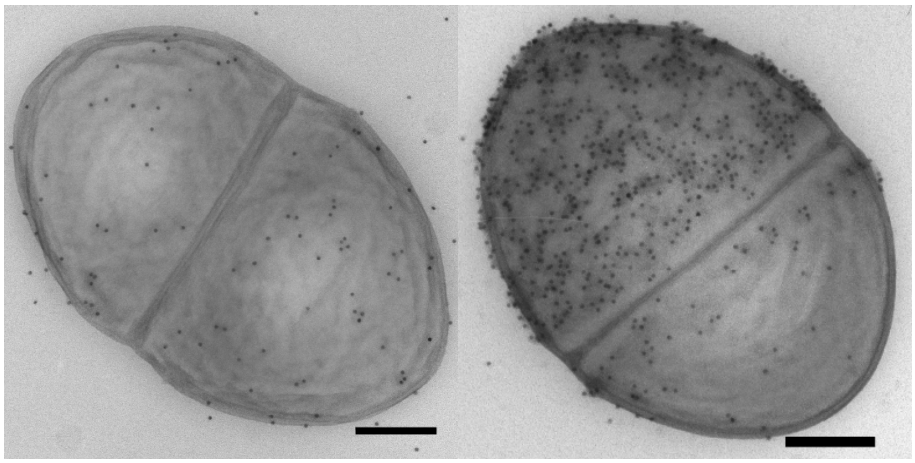
Figure 4.16: Differential expression of cell wall-associated Esp at 37 °C and 21 °C measured by flow cytometry (FACS) of anti-Esp labelled cells. Strains UW3114 (donor) and MMH594 (reference) are shown for comparison. Strains 64/3 T-10 (64/3xUW3114 T-10) and OG1RF T-12 (OG1RFxUW3114 T-12) are the transconjugants that acquired the *E. faecalis* PAI from strain UW3114 (See also Table 3.9). Control is a conjugate control. The mean values from two different experiments are shown, error bars denote standard deviations.

4.6.2 Biofilm formation

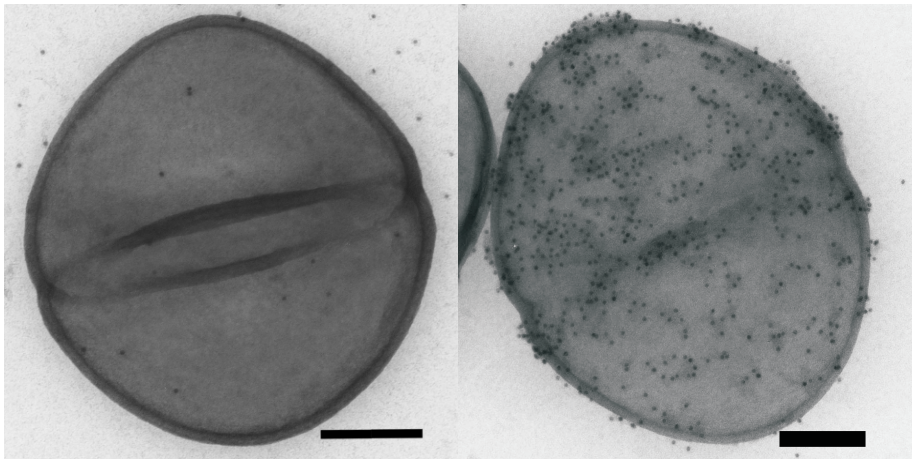
The *E. faecalis* transconjugant strain OG1RFxUW3114 T-12 showed a 2-fold increase in biofilm forming capacity compared to its homologous strain, the recipient OG1RF. In the *E. faecium* strains both the recipient and the transconjugants showed very low biofilm formation (Figure 4.18).

4.6.3 Cytolysin/haemolysin

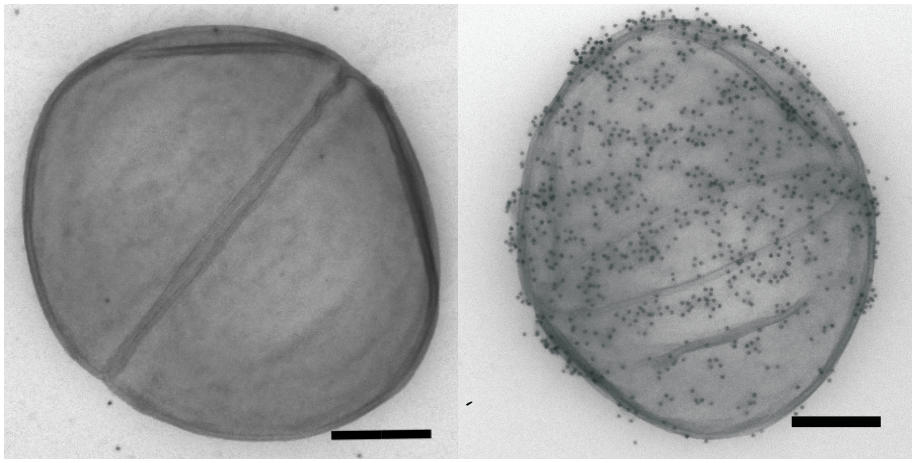
Both the reference (MMH594) and the donor (UW3114) strains showed haemolytic activity while none of the recipient strains were haemolytic. The *E. faecalis* transconjugant developed a strong beta-haemolysis after PAI acquisition, while the pathogenicity island (PAI) positive *E. faecium* transconjugant remained non-haemolytic. Since the cytolysin operon can also be plasmid-encoded, haemolysis was also tested in transconjugants carrying the *erm*(B) plasmid pLG2 but lacking the PAI and were non-haemolytic. Furthermore, sequencing of pLG2 did not reveal the presence of any haemolysin or cytolysin genes (see below). Consequently the acquired haemolytic activity of *E. faecalis* transconjugants could be linked exclusively to the cytolysin operon present within the PAI.



(a) MMH594 (b) UW3114



(c) 64/3 (d) 64/3xUW3114 T-10



(e) OG1RF (f) OG1RFxUW3114 T-12

Figure 4.17: Esp expression shown by transmission electron microscopy of cells negatively stained and labeled with immunogold (15 µm) using anti-Esp antiserum. Strains (a) MMH594 (reference) and (b)UW3114 (donor) are shown for comparison. Strains (d)64/3xUW3114 T-10 and (f)OG1RFxUW3114 T-12 are the transconjugants that acquired the plasmid pLG2 and the *E. faecalis* PAI from strain UW3114 (See also Table 3.9). Bar length= 200 nm

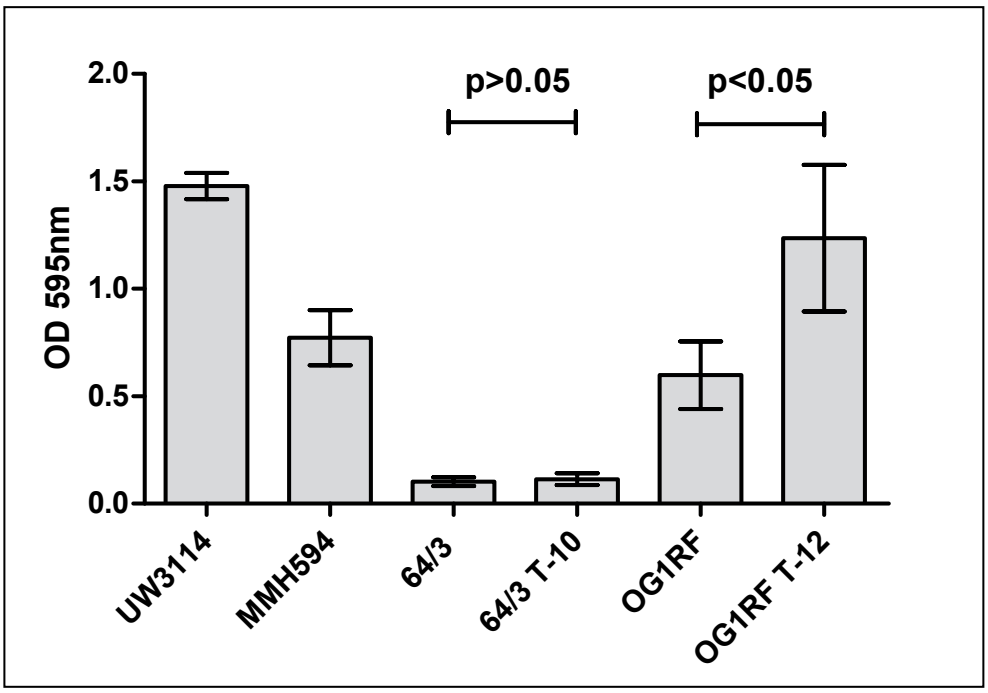
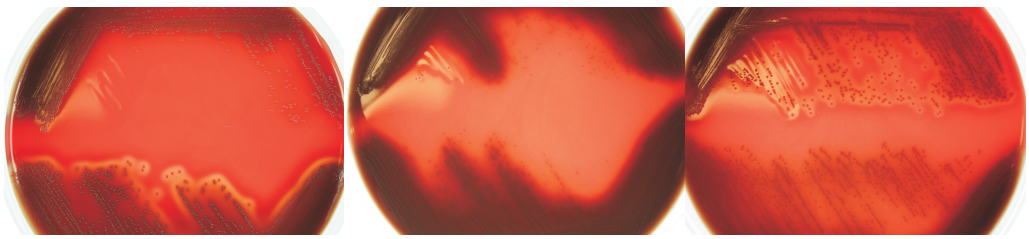


Figure 4.18: Comparison of *in vitro* biofilm formation of the transconjugants. Strains UW3114 (donor) and MMH594 (reference) are shown for comparison. Strains 64/3 T-10 (64/3xUW3114 T-10) and OG1RF T-12 (OG1RFxUW3114 T-12) are the transconjugants that acquired the plasmid pLG2 and *E. faecalis* PAI from strain UW3114 (See also Table 3.9). The mean values of three different experiments are shown. Error bars denote standard deviation.



(a) (b) (c)

Figure 4.19: haemolytic activity tested on human blood agar plates. (a) top, non-haemolytic recipient strain OG1RF; bottom, haemolytic donor strain UW3114. (b) Non-haemolytic *E. faecium* transconjugant strains: top, 64/3xUW3114 T-10 bearing PAI, bottom, 64/3xUW3114 T-1 PAI free. (c) *E. faecalis* transconjugant strains: top, haemolytic OG1RFxUW3114 T-12 bearing PAI, bottom non haemolytic OG1RFxUW3114 T-1 PAI free.

4.6.4 Animal experiments

Mouse bacteraemia and peritonitis models were used to compare the pathogenic potential of *E. faecalis* isogenic strains differing only by presence of the PAI. The bacterial loads in blood and kidneys after 24 h of infection were compared. The differences between the PAI-positive and PAI negative transconjugant strains were not statistically significant. However, the PAI-positive strain yielded lower bacterial counts than its isogenic strain lacking the PAI although the bacterial counts in blood in the bacteraemia model were higher. (Figure 4.20). The mouse bacteraemia model was used to compare the pathogenicity of the *E. faecium* isogenic strains carrying and lacking the PAI. Although not significant, the *E. faecium* transconjugant strain bearing the PAI 64/3xUW3114 T-10 appeared to yield lower bacterial counts than its corresponding recipient and PAI negative isogenic strain (64/3xUW3114 T-1) (Figure 4.20). However the inoculum of the PAI positive *E. faecium* strain was ca. 1 log lower, not allowing any possible conclusions.

4.6.5 *in vitro* Growth

Growth of the recipient strains and the transconjugant strains lacking and carrying the PAI was compared in order to determine differences in growth as a consequence of the altered genome size (ca. 266 kb larger genome in PAI-positive strains). There was no significant difference for the *E. faecium* recipient vs. transconjugant strains ($p>0,05$). In *E. faecalis* a significant delay of growth was observed in the PAI-carrying transconjugant compared to the recipient from the beginning of the log phase ($p<0,05$) (Figure 4.21).

4.6.6 Adherence to epithelial cells

Differential adherence to epithelial cells *in vitro* of the transconjugant strains lacking and carrying the PAI was compared using CACO cells. The differences observed did not reach statistical significance, although both *E. faecalis* and *E. faecium* transconjugant strains carrying the PAI were slightly more adherent than those lacking the PAI (Figure 4.22)

4.7 Plasmid pLG2 sequencing

The plasmid pLG2 (calculated size 66 kb) was sequenced in order to investigate the presence of gene clusters that might be involved in conjugation and could have supported the horizontal transfer of the *E. faecalis* PAI. The sequences of pLG2 can be found in GenBank under the accession numbers HQ426650 to HQ426665. Only contigs larger than 400 bp and with identified ORFs were submitted (52 kb). The assembly statistics after 454-sequencing of pLG2 were the following:

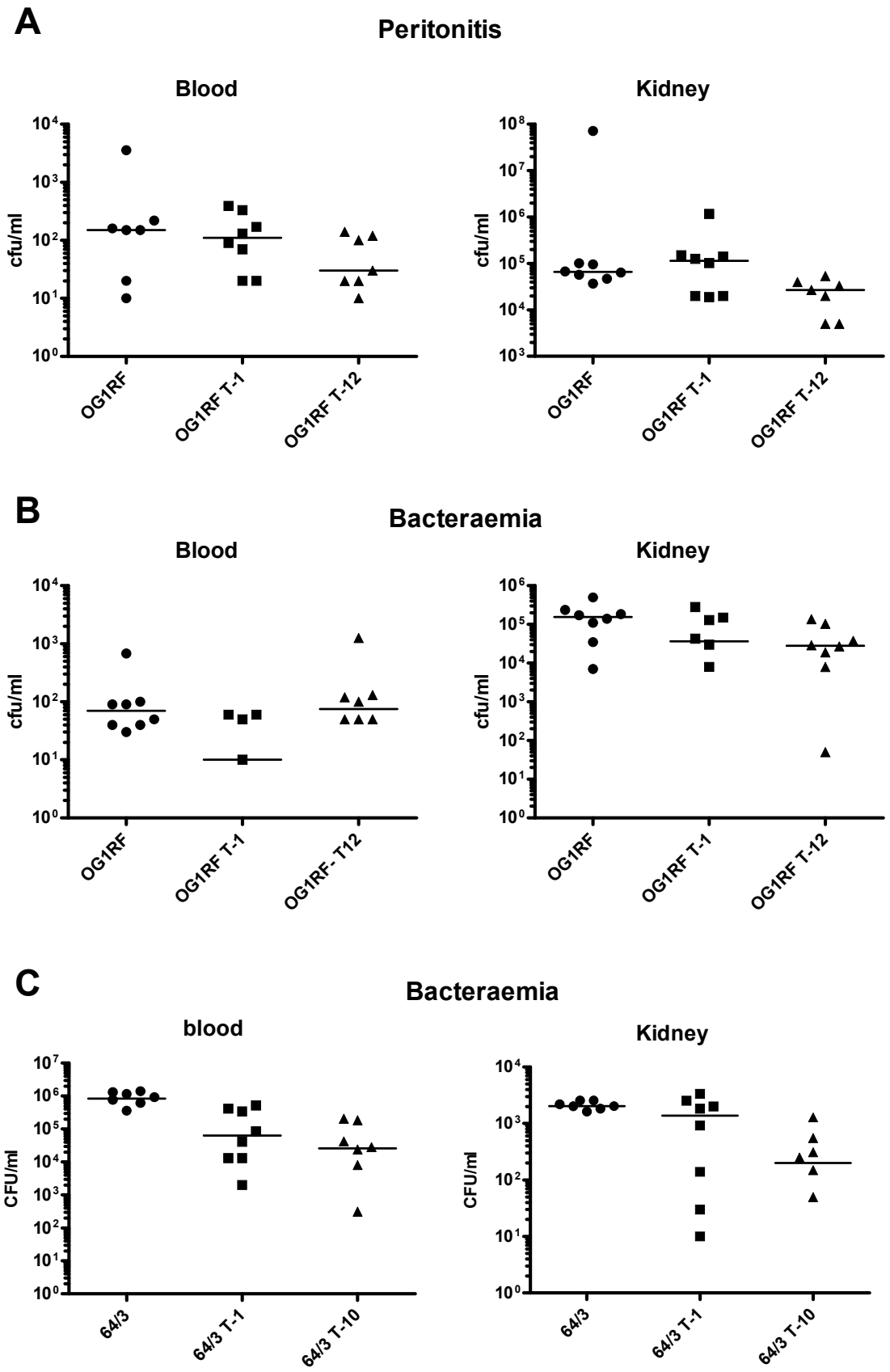


Figure 4.20: Bacterial counts in the blood (left) and kidneys (right) 24 h after A, intraperitoneal, or B, intravenous, injection of 6-8-week-old female BALB/c mice with *E. faecalis*. C, shows results after intravenous injection with *E. faecium* strains. Data represent the individual bacterial counts and the media. The inocula used for the bacteraemia model were 64/3: 9.6×10^8, 64/3xUW3114 T-1: 1.26×10^9, 64/3xUW3114 T-12: 2.8×10^8, OG1RF: 8.5×10^8, OG1RFxUW3114 T-1: 5.5×10^8, OG1RFxUW3114 T-12: 5.2×10^8. The inocula used for the peritonitis model were OG1RF: 4.4×10^8, OG1RFxUW3114 T-1: 5.3×10^8, OG1RFxUW3114 T-12: 5.7×10^8.

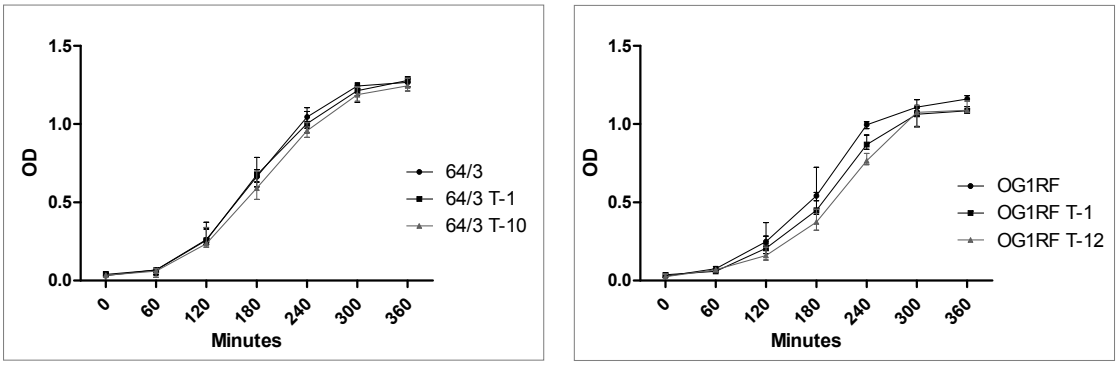


Figure 4.21: Comparative *in vitro* growth of *E. faecium* (left) and *E. faecalis* (right) recipient and transconjugant strains. Strains 64/3 T-10 (64/3xUW3114 T-10) and OG1RF T-12 (OG1RFxUW3114 T-12) are the transconjugants that acquired pLG2 and the *E. faecalis* PAI from strain UW3114 (See also Table 3.9). Error bars denote standard deviation.

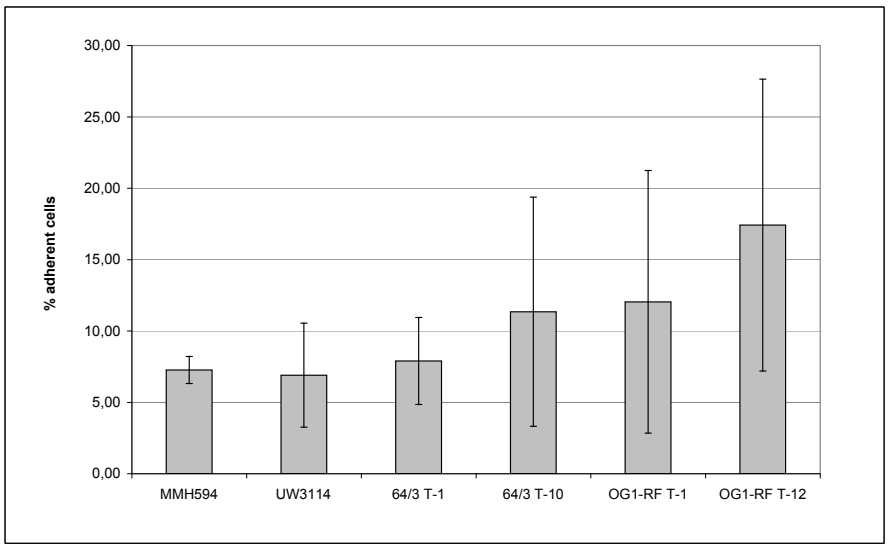


Figure 4.22: Comparison of *in vitro* adherence of the transconjugants to CACO-2 cells. Strains UW3114 (donor) and MMH594 (reference) are shown for comparison. Strains 64/3 T-10 (64/3xUW3114 T-10) and OG1RF T-12 (OG1RFxUW3114 T-12) are the transconjugants that acquired the plasmid pLG2 and *E. faecalis* PAI from strain UW3114. The mean values of three replicates are shown. Error bars denote standard deviation.

- total of contigs: 46
- total number of aligned bases: 62.6 kb
- contigs lager than 500 bp: 17
- number of bases in contigs larger than 500 bp: 53.2 kb.
- average contig size: 1362 bp
- largest contig: 33,447 bp

Analysis of the plasmid sequences showed that pLG2 does not correspond entirely to any previously described enterococcal plasmid. Similarities to fragments of different enterococcal plasmids (pAD1, pTEF1, pTEF2, pAM373, pVEF1, pVEF2, pEF1, pIP816, pRE25) were discovered, especially to putative genes encoding replication and mobilization functions suggesting that pLG2 has a modular structure. Complete replication and mobilization modules including an *oriT* suggest that pLG2 is a conjugative plasmid, theoretically capable of triggering the transfer of the *E. faecalis* PAI. The finding of a pheromone binding protein and the structure of the replication module suggested that pLG2 is a pheromone responding plasmid. Determinants encoding antibiotic resistance to erythromycin, streptomycin and streptothricin were also located within the plasmid sequence (Table 4.8).

We identified a replicase gene, repR-pLG2 (pLG2-0001) that shared 100% identity at the nucleotide level to the replicase genes of *E. faecium* plasmids p5753cB (GQ900487.1), pIP816 (AM932524.1), pVEF1-3 (AM296544.1, AM410096.1, AM93 1300.1), pEF1 (DQ198088.1) and *E. faecalis* plasmid pRE25 (X92945.2) representing replicases of Inc18-type plasmids. Similarity extended to a 5.3 kb region spanning the replicase (contig 13 coordinates 1:5374). The region spanning the replicase gene was 99% identical to the 3.7 kb replication region of pEF1, which is virtually identical to a homologous region described in pRE25. This region contains a putative replication origin *oriR*, up to five DnaA boxes and several inverted repeats [127]. With regard to the segregation machinery, we identified a putative PrgP-PrgO partitioning system, upstream and inverted to the replicase gene.

The region between coordinates 2783:1091 of contig 8 shared 98% similarity at the nucleotide level along 1.6 kb of the 4.8 kb mobilization region of pEF1 (e=0.0). A resolvase (pLG2-0012) and a "Filamentation induced by cAMP protein-Fic" (pLG2-0013), homologous to pEF1 MobC are encoded in this region. Within the same contig, an origin of transfer (*oriT*) was identified based on the 99% similarity (expect=0.0) to the mobilization region of pAD1 where an *oriT* has been previously identified between ORF53 and ORF57 (AF343837) [38]. No ORF that exhibits homology to a relaxase could be identified. All the elements necessary for plasmid mobilization in Gram-positives were identified in pLG2 as can be seen in Table 4.8. Other mobilization related genes of pLG2 include a putative single strand binding protein gene (pLG2-0026) that could act as a coupling protein that binds the DNA for substrate presentation during transfer, two peptidase putative genes (pLG2-0058

and pLG2-0059), which might degrade the bacterial cell wall for the formation of a transfer canal and two putative resolvase genes (pLG2-0012 and 38) that could be involved in plasmid segregation by resolving plasmid dimers.

Locus Tag	Contig	Accession number	Coordinates	Product
Replication and mobilization				
pLG2-0001	13	HQ426650	1975 3468	similar to plasmid replication protein [*E. faecium* DO]
pLG2-0003	13	HQ426650	4068 5033	PrgP [*E. faecium*]
pLG2-0004	13	HQ426650	5005 5280	PrgO protein [*E. faecalis* TX1322]
pLG2-0063	25	HQ426655	2 373	SPOUT methyltransferase superfamily protein [*E. faecalis* ATCC 29200]
pLG2-0065	27	HQ426657	3 506	Nucleotidyltransferase/DNA polymerase involved in DNA repair Enterococcus sp. 7L76]
pLG2-0012	8	HQ426665	1489 2061	resolvase [*E. faecalis* TX0104]
pLG2-0013	8	HQ426665	2077 2682	Filamentation induced by cAMP protein Fic [*E. faecium* DO]
pLG2-0019	8	HQ426665	7519 8400	DNA nuclease [*E. faecalis* TX0104]
pLG2-0026	8	HQ426665	11240 11716	single-strand binding protein [*E. faecalis* V583]
pLG2-0027	8	HQ426665	11856 13190	PcfJ [*E. faecalis* JH1]
pLG2-0038	8	HQ426665	24043 24597	resolvase [*S. pneumoniae* CGSP14]
pLG2-0047	8	HQ426665	30710 32254	DNA recombinase, putative [*Streptococcus suis* 05ZYH33]
pLG2-0048	8	HQ426665	32274 32690	recombinase [*Streptococcus suis* 05ZYH33]
pLG2-0049	8	HQ426665	32691 33017	possible DNA recombinase [*E. faecalis* TX0104]
pLG2-0050	8	HQ426665	33021 33188	DNA recombinase, putative [*Streptococcus suis* 05ZYH33]
pLG2-0058	17	HQ426652	2 133	peptidase [*E. faecalis* T1]
pLG2-0059	17	HQ426652	126 461	peptidase M16 [*E. faecalis* T1]
pLG2-0067	30	HQ426659	3 338	cell wall surface anchor family protein [*E. faecalis* TX0104]
pLG2-0068	30	HQ426659	447 677	cell surface protein [*E. faecalis* T2]
pLG2-0072	38	HQ426663	14 481	pheromone binding protein [*E. faecalis* TUSoD Ef11]
pLG2-0069	31	HQ426660	3 578	phage infection protein [*E. faecalis* HIP11704]
Antibiotic resistance				
pLG2-0039	8	HQ426665	24952 25689	erythromycin resistance transferase [*E. faecium*]
pLG2-0040	8	HQ426665	25790 26155	predicted protein [*E. faecalis* T2]
pLG2-0041	8	HQ426665	26439 27233	aminoglycoside 3'-phosphotransferase [*Staphylococcus epidermidis* RP62A]
pLG2-0042	8	HQ426665	27326 27481	streptothricine-acetyl-transferase Campylobacter coli]
pLG2-0043	8	HQ426665	27552 27794	possible streptothricin acetyltransferase [*E. faecalis* TX0104]
pLG2-0044	8	HQ426665	27803 28711	streptomycin aminoglycoside 6-adenyltransferase [*E. faecium*]
pLG2-0056	14	HQ426651	1664 3604	tetracycline resistance protein [*Lactococcus garvieae*]
others				
pLG2-0060	20	HQ426653	15 503	FeS assembly protein sufD [*E. faecalis* T11]
pLG2-0061	24	HQ426654	10 288	regulator of sorbitol operon [*E. faecalis* TX1322]
pLG2-0062	24	HQ426654	340 588	transcriptional regulator SrlR [*E. faecalis* V583]
pLG2-0064	26	HQ426656	2 502	aspartate kinase [*E. faecalis* E1Sol]
pLG2-0066	29	HQ426658	2 481	GTP-binding protein [*E. faecalis* T3]
pLG2-0073	43	HQ426664	2 169	RinA family transcriptional regulator [*E. faecalis* V583]

Table 4.8: Annotation of complete identified ORF in the sequencing contigs of pLG2. Contigs where the ORF is found and exact location within them are given.

5. Discussion

5.1 hyl_{Efm} Genomic environment

The putative hyl_{Efm} genomic island (GI) was reannotated as a 17,817 bp structure highly conserved among hyl_{Efm} positive *Enterococcus faecium*. This high conservation was seen when analyzing nucleotide databases and by investigating a set of 39 diverse clinical hyl_{Efm} positive strains. 20 of these strains appeared to have an insertion downstream of hyl_{Efm} at a specific region (hylGI3c) (Table 4.3). The prevalence of this event among the strains analyzed can be biased by over representation of hyl_{Efm} positive isolates recovered during 2006 and the prevalence of this insertion among hyl_{Efm} positive strains worldwide remains unclear. In pLG1 this insertion was resolved as a 2,344 bp, constituting a relic of IS*66* (Table 4.4, Figure 4.5). Recently Gourbeyre *et al.* have described that IS*66* is a family of ISs largely limited to the Proteobacteria, which normally include three ORFs (*orfA*, *orfB* and *orfC*), well conserved terminal inverted repeats of about 20-30 bp and are flanked by an 8 bp direct repeat [44]. IS*66* of pLG1 consists of the transposase (*orf*C) and what seems to be the *orf*A component. pLG1 bears the 25 bp inverted repeats with highly conserved sequence described by Gourbeyre *et al.* (GTAAGCGNCCNYN) but the 8 bp direct repeats at the insertion site are not present. IS*66* family ISs are largely limited to the proteobacteria, but include two well-defined clades, one limited to the Bacteriodetes/Chlorobi (green sulfur bacteria) and the other one to the Firmicutes [44]. Whether the IS*66* transposase of pLG1 is functional, cannot be told. Functionality of IS*66* transposase has been confirmed only for IS*679* by Han *et al.* [49], however pLG1's IS*66* has no significant similarity to IS*679*, but to other regions annotated as IS*66* from enterococci and lactobacillae. hyl_{Efm} GI encodes a two component system transcription regulation operon that could regulate the expression of the hyl_{Efm} GI-genes. Two component systems are commonly used by pathogenic bacteria to control the expression of virulence factors required for infectivity [157].

hyl_{Efm} was found to be plasmid located in 37 (n=39) *E. faecium* strains (plasmid sizes 150 to 350 kb). *vanA* was co-localized in the hyl_{Efm} plasmid in 4 isolates (n=12). The co-localization of *vanA* and hyl_{Efm} onto the same plasmid has also been reported by Arias *et al.* and Rice *et al.* [3, 121]. Freitas *et al.* analysed a set

of 51 hyl_{Efm} positive isolates from a worldwide collection of vancomycin-resistant and vancomycin-susceptible enterococci belonging to the clonal complex (CC) of hospital associated strains (CC17). They found that hyl_{Efm} was located on plasmids ranging from 170 kb to 375 kb and in contrast to the results presented here, *vanA* and hyl_{Efm} genes were never identified on the same plasmids [39]. The different sizes of the hyl_{Efm} megaplasmids suggests different structures and compositions, however further differences or similarities were not investigated. Among the strains analyzed the isolates of the year 2006 representing ST-192 (MLVA-159) isolates might be over represented more likely due to a clonal spread than to an increase in hyl_{Efm} positive strains during that period [also discussed in 191]. hyl_{Efm} appeared to be chromosomally located in strains DO and UW6379, although in previous studies it has been reported that hyl_{Efm} is located on a plasmid in strain DO [3, 121]. Probably propagation, several passages and manipulation of this strain in different laboratories over years, turned it into a descendent of the original strain DO in which hyl_{Efm} is now integrated in the chromosome. It is worth mentioning that strain UW6379 was the only strain where the region downstream of hyl_{Efm} the putative GI structure was completely absent (Table 4.3).

None of the 39 strains tested in this study showed any hyaluronidase activity. Original annotation of hyl_{Efm} was based on translated nucleotide similarity (BlastX) to a hyaluronidase from *Streptococcus pyogenes* known as Spy1600 (Accession numbers NP_269657 and NP_607664) and to a hyaluronidase from *Clostridium perfringens* [120]. A recent report demonstrates that Spy1600 despite its original annotation as a hyaluronidase does not have detectable hyaluronidase activity but does remove ß-O-linked N-acetylglucosamine from mammalian glycoproteins [146]. This might also be the case of hyl_{Efm}, however no biochemical evidence for the actual enzymatic activity of hyl_{Efm} has been reported so far. The predicted hyl_{Efm} protein sequence did not reveal any protein domains or functional sites that allowed prediction of an alternative function. ORFs adjacent to *spy1600*, as those adjacent to hyl_{Efm}, encode a transcriptional regulator, other glycosidases and sugar binding proteins supporting the idea that these genes are involved in carbohydrate metabolism although a role in deglycosylation of human O-glcNAc proteins cannot be ruled out [146]. Blast comparison of the hyl_{Efm} GI and a 20 kb region spanning *spy1600* showed no similarity between these two regions except for the hyl_{Efm} gene and the region encoding two binding protein dependent transport systems (pLG-037 and pLG-038) (64% identities). However the genomic region spanning *spy1600*, similar to hyl_{Efm} GI, bears genes related to transcription regulation, carbon metabolism and sugar transport systems (Figure 4.5, Table 4.4). Specific metabolic activities have been reported as plasmid-encoded, e.g. Zhang *et al.* reported recently the link of alpha-galactosidase to conjugative megaplasmids in *E. faecium* [202]. It is possible that the exclusive association of hyl_{Efm} to hospital associated strains is due to the antibiotic selective pressure imposed in the clinical settings that favors the spread

and maintenance of hyl_{Efm} multiresistance plasmids on the population.

5.2 hyl_{Efm}-Megaplasmid (pLG1)

Large plasmids have not been previously described in enterococci [186], however according to recent reports it seems like they are widely disseminated [39, 179]. The sequence of plasmid pLG1 is the first reported sequence of a megaplasmid in *E. faecium*. Several molecular and epidemiological studies have revealed the exclusive prevalences of hyl_{Efm} among clinical strains [120, 173]. In this study, however, the in vitro transfer of hyl_{Efm} plasmids was demonstrated from various clinical *E. faecium* strains into clinical and non-clinical *E. faecium* recipient strains of different MLST types (Table 3.8). The fact that hyl_{Efm} plasmids were easily transferable into non-hospital recipient strains of different MLST types means that a narrow host range of the hyl_{Efm}-plasmids can not be responsible for the exclusive presence of hyl_{Efm} among clinical strains. The transfer of hyl_{Efm} plasmids into *E. faecalis* could not be achieved, which is in accordance to the fact that this marker is exclusively found among *E. faecium* [120] suggesting a limited host range of the hyl_{Efm} megaplasmids as a reason for this exclusive distribution. Horizontal transmission of hyl_{Efm} plasmids has been previously described both when hyl_{Efm} and *van* are genetically linked [3, 121] and when they are not [39]. It is known that plasmids are dynamic structures that change by gain and loss of genomic fragments influenced by the changing environment in which they reside [71]. Differences observed among the transconjugants can be due to rearrangements and duplications of genomic fragments or incomplete transfer during plasmid conjugation as has been also observed by others in various plasmid conjugation experiments (Freitas, A und Coque, T. personal communication). At the beginning of this study hyl_{Efm} had only been described as a genomic marker and no plasmid location had been suggested [120]. After the first conjugation experiments were performed and high transfer rate of hyl_{Efm} was observed, the genomic location of hyl_{Efm} was investigated and it was found that hyl_{Efm} was encoded within large plasmids. Shortly after, the plasmid location of hyl_{Efm} was reported [3, 121]. Other enterococcal virulence factors are also present into plasmids. These are the *E. faecalis* agregation substances ASA1 and Asc10 (adherence and internalization to human cells) [112, 16, 119, 182, 158], the ebp locus (biofilm, endocarditis and UTI- associated pili of *E. faecalis*) [107, 150] and the cytolysin (increased mortality in endocarditis model) [16].

The presence of several insertions sequences, transposases and other repetitive elements along plasmid pLG1 complicated the assembly of the 454-generated sequences but at the same time, together with the presence of phage-related genes and recombinases, supported the current model that plasmids in *E. faecium* are mosaic-like elements.

A putative replicase gene *repA*-pLG1 was identified with a conserved *oriV*, based on the similarity to the RepA of the *E. faecalis* plasmid pAD1. Francia *et al.* have demonstrated that the replication initiator protein of pAD1, RepA, is necessary and sufficient for initiation of plasmid DNA replication at an *oriV* located within its own coding sequence, based on its ability to support replication when RepA was supplied in trans [37]. RepA of pAD1 functions as the replication initiator protein [185]. The C-terminal sequence of RepA-pLG1 has a low similarity (30%) to that of RepA_pAD1, while the N-terminal sequence had high similarity (79%). The C-terminal sequence of RepA_N homologs is highly variable and provides variability and the host specificity [185]. The specificity provided by the C-terminal sequence of RepA-pLG1 could explain why hyl_{Efm} is never found among *E. faecalis* as well as the negative results in hyl_{Efm} plasmids transfer into *E. faecalis*. The N-terminal sequence similarity between RepA-pLG1 and RepA_pAD1 matches to the observation that most sequence conservation among RepA homologs is found at the N-terminal region (first 100 amino acids of each protein), which constitutes the RepA_N conserved domain [185]. Most members of the RepA-pAD1 related family are similar in size [37]. However RepA-pLG1 is larger than other enterococcal RepA. Weaver *et al.* constructed an un-rooted phylogenetic tree of 43 RepA homologs associated to mobile genetic elements to demonstrate the broad distribution and narrow host range of RepA_N plasmids [185]. This phylogenetic analysis applied to the RepA-pLG1 confirmed that it is a RepA_N family replicon. The RepA_N replicons represent an ancient group of plasmids that have co-evolved with the low G + C Gram-positive bacteria. The replication, partition and conjugative transfer components of the RepA_N plasmids have not evolved as a single unit but rather as separate modules that have been shuffled among various plasmids native to these organisms [185]. A putative *repB* was identified in pLG1. RepB proteins are ATPases that, together with a DNA binding protein and a centromere-like site, comprise an "active partition locus" which facilitate plasmid distribution at cell division [185]. Different to RepA, RepB family of proteins are not associated with the RepA_N family of replicons and its comparison fail to segregate by host genus as do the RepA_N proteins [185]. The putative *repB*-pLG1 has homology to proteins related to CDS1_pRE25 which do not belong to RepA_N family of replication initiators, indicating that replication and maintenance are two different plasmid modules. This matches to the observation that RepA_N plasmids show an independent evolution for replication and maintenance modules. Within *Enterococcus*, RepB homologs of several Gram-positives are interspread evidencing the modular evolution of these plasmids [185]. A putative relaxase gene was identified. Relaxases are the proteins that initiate and terminate conjugative DNA processing and are the only essential protein of the mobilization (MOB) machinery of mobilizable plasmids [40]. Garcillán-Barcia and collaborators have described a classification scheme of conjugative transfer systems based on the comparison of the sequences and properties of the relaxase proteins contained in

gene sequence databases [40]. This analysis applied to Rel-pLG1 showed that it belongs to the MobP7 subfamily of the largest relaxase family MobP. MobP7 relaxases have been found encoded by RepA_N plasmids before (e.g. pCF10); pAD1, instead, encodes a MobC relaxase [40]. PCR investigation of backbone genes of pLG1 in the 39 hyl_{Efm} positive strains indicated that the *repA*-pLG1 was always present, while the relaxase, resolvase and mobC, were variably present. However, hyl_{Efm} negative strains carrying large sized plasmids also carry *repA*-pLG1, suggesting that this replicase is widely distributed (Table 4.5) and not exclusively associated to hyl_{Efm} presence. Although not confirmed by Southern hybridization it is feasible that the *repA*-pLG1 is the replicase of hyl_{Efm}- megaplasmids, considering that in a previous study, none of the until then known Gram-positive replicase genes could be found in hyl_{Efm}- megaplasmids (n=51) [39].

Hendrickx *et al.* have previously described 4 different pilin gene clusters (PGC) in *E. faecium* [58]. Pili are supposed to be implicated in adhesion to multiple types of human cells and in biofilm formation [reviewed in 136]. In *E. faecalis* pili are reported to be associated with biofilm formation playing a role in endocarditis and urinary tract infections [150, 149]. A complete copy of PGC-1 with all the conserved motifs of the described pilin subunits was found in pLG1. The major pilin subunit of *E. faecium* PilA has very conserved pilin (LxxIHLYPKNxx) and E box (YxxxETxAPExY) motifs, as well as an N-terminal signal peptide sequence and an LPXTG-like sortase substrate motif at the C-terminus [59]. Meanwhile the minor pilin subunit PilF contains a variant pilin motif, an N-terminal peptide but not a cell wall anchor and PilE contains only an N-terminal signal sequence and a putative cell wall anchor motif [58]. Originally the genomic localization of these PGC was not studied in detail [59]. A recent study demonstrated the plasmid co-localization of *pilA* (*fms21*) and hyl_{Efm} in 3 of 7 clinical *E. faecium* isolates [73]. speculated that ancestral *E. faecium* isolates carried the *pilA* gene cluster and subsequently acquired antibiotic and virulence determinants.

Conjugation systems appear to be a subgroup of T4SS that have evolved the additional capacity to translocate DNA-protein complexes [17]. In Gram-positive plasmids, proteins homologous to most of the protein families involved in DNA transfer and in Gram-negative bacterial plasmid transfer have been described [reviewed in 46]. These should provide:

- substrate presentation (coupling proteins or VirD4): coupling proteins that are thought to link the DNA transfer intermediate and perhaps guide it through the mating channel,
- necessary energy for the translocation process (ATPases or VirB4, VirB11 homologs): mainly ATPases that might act as chaperones involved in translocation of transfer components across the membranous system and
- formation of the mating channel (lytic transglycosylases or VirB1 homologs

and transmembrane proteins or VirB6 homologs): homologs to the family of lytic transglycosilases that might open the peptidoglycan and aid the DNA and proteins to cross the cell envelope- lysozyme.

In Gram-positive bacteria the close contact between donor and recipient cells preceding conjugative transfer is thought to be established without the help of pili [2] and is supposed to involve the role of adhesins, aggregation substance or proteins with cell wall anchor motifs. No association between pili and DNA/protein secretion or transfer has been assessed in Gram-positive bacteria. Nevertheless Type IV pilin-like protein genes have been demonstrated to be involved in genetic transformation of Gram-positive bacteria [reviewed in 5] and genes coding for secretion channel-like proteins have been described for several Gram-positive conjugative plasmids [reviewed in 15]. Hence participation of pilus-like structures in the conjugative transfer processes of Gram-positive bacteria cannot be excluded [2]. Presence of genes required for mobilization as well as a T4SS allowing assembly and functionality of the mating channel are hallmarks of self-transmissible plasmids [40]. In the vicinity of this region, an ORF (pLG-0015) with similarity to a single stranded DNA (ssDNA) binding protein (SSBP) was found which is normally encoded in the conjugation regions of some sex pheromone plasmids such as pAD1. SSBPs are believed to coat the ssDNA in order to protect it from the action of nucleases in the recipient cell. In pLG1 a set of genes were found that resemble a T4SS operon.

Plasmids possess a number of systems that, by virtue of their ability to kill cells which have lost a specific plasmid element, stabilize the plasmid in a growing population of cells. There are two types of stabilization systems that kill plasmid-free cells. In one case, both toxin and antitoxin proteins are specified by the plasmid but the toxin protein is more stable, or less susceptible to degradation than is the antitoxin protein (TA systems); in the other case the synthesis of the toxin protein is prevented by inhibition of translation by a plasmid-specified antisense RNA. Several identified ORFs of pLG1 seem to be involved in plasmid maintenance. A putative TA module was identified, which shows the characteristic size and overlapping organization observed in proteic TA modules found in Gram-negative bacteria [45] and in the *E. faecium* plasmid pRUM [45]. TA systems have been described in a variety of enterococcal plasmids [187, 153].

Bacteria have developed homeostatic mechanisms to ensure adequate levels of copper within the cell. However increased concentrations of copper are toxic, in response to which plasmid-borne copper resistance mechanisms, such as the *tcrYAZB* operon of *E. faecalis* and *E. faecium*, are often employed [51]. The finding of a complete *tcrYAZB* copper resistance operon in pLG1, among other putative heavy metal resistance genes, corroborates previous studies describing the presence of copper resistance, glycopeptide and macrolide resistance gene clusters on the same conjugative plasmid [52]. The identified complete *vanA* operon of pLG1 constitutes a subtype of

Tn*1546*-like elements which often bear IS*Ef1*, IS*1678*, IS*1216V*, IS*Efa5*, IS*19*-like between *vanX* and *vanY* [190, 72, 13, 87].

Several putative genes of pLG1 seem to be involved in carbohydrate metabolism. Several PTS components were identified, always in the vicinity of metabolic genes. PTS constitute the major carbohydrate transport system in bacteria by active translocation across the cell membrane. PTSs catalyze the transfer of the phosphoryl group from phosphoenolpyruvate to incoming sugar substrates concomitant with their translocation across the cell membrane. In addition to catalyzing sugar transport, the PTS is involved in regulation of non-PTS transport, carbon and nitrogen metabolism, chemotaxis and other processes [116, 125]

5.3 Intra and inter species transfer of the *E. faecalis* pathogenicity island (PAI)

The results presented in this work demonstrate for the first time that the entire *E. faecalis* pathogenicity island (PAI) is capable of precise excision, circularization and horizontal intra- and interspecies transfer. Chromosome-to-chromosome transfer and site-specific integration of the *E. faecalis* PAI into the chromosome of *E. faecalis* and *E. faecium* were demonstrated. The *E. faecalis* PAI is flanked by 10 bp direct repeats (DR) and the first two genes encoded within the PAI are a putative phage-related integrase and an excisionase [141] suggesting that it can excise from and integrate into the chromosome by homologous recombination and can be transferred as a single entity [22]. The *E. faecalis* strain that was used as donor (UW3114) had been successfully used by Oancea *et al.* to demonstrate the intra-species transfer of the *esp* gene into *E. faecalis* [111]; in that study, the transfer of further fragments of the PAI could not be investigated in detail because the recipient strain *E. faecalis* JH2-2 already possesses parts of the PAI. Coburn *et al.* have described excision of a 27,744 bp internal fragment of the PAI, circularization, integration into a pTEF1-like plasmid and transfer into an *E. faecalis* recipient strain [22]. Recently Manson *et al.* have shown that the *E. faecalis* PAI can transfer into *E. faecalis*, not precisely but as part of larger chromosomal fragments. The horizontal transfer of such regions requires the help of either pTEF1 or pTEF2 pheromone-responsive plasmids and is *recA*-dependent, while the integrase and excisionase genes present in the PAI were not required [89]. However, a role of the integrase and excisionase genes for the precise excision and integration of the PAI cannot be ruled out. This is the first report of the transfer and presence of the *E. faecalis* PAI into *E. faecium*. van Shaik *et al.* have recently indicated that some hospital strains of *E. faecium* carry a variant of the *esp* gene (esp_{Efm}) in a PAI- like structure different from the *E. faecalis* PAI. The esp_{Efm} PAI is comprised along *E. faecium* U0317 Contig 00248 ($gb|ABSW01000226.1|$) and contains a 10 kb

fragment identical to a gene cluster in the *E. faecalis* PAI that suggests a recent intra-genus transfer or acquisition of the PAIs from a third source [179]. van Schaik *et al.* state that the esp_{Efm} PAI can be transferred horizontally among *E. faecium* [179]. Given the species-specific distribution of these two elements it is possible that the *in vitro* inter-species transfer of the *E. faecalis* PAI is a less frequent event *in vivo*.

The analysis of the PAI structure in the donor and the transconjugants revealed that it did not undergo any changes during transfer. Compared to the originally described PAI of strain MMH594, the transferred PAI structure contained some deletions and insertions (Figure 4.12). The differences observed along the transferred PAI compared to the prototype PAI of strain MMH594 agrees with the previous observation that the gene content of the *E. faecalis* PAI is highly variable and PAI subtypes are widely spread among different sequence types, clonal types and strains of various clinical and non-clinical origin [90, 143, 91]. The cluster-like variability in gene composition and presence of sequences related to mobile genetic elements within the PAI suggest that it has evolved by initial spread of some core elements and later recombination, acquisition and loss of gene clusters [90, 91].

The *E. faecalis* PAI is integrated into a non-coding region flanked by an ORF specifying a hypothetical protein with no predictable function and a putative oxidoreductase [141]. The *E. faecalis* PAI of the *E. faecalis* donor strain UW3114 was located into the same region and it also integrated into this region in the recipient strain OG1RF, suggesting a highly site-specific integration. The *E. faecium* recipient strain 64/3 lacks the esp_{Efm} PAI. In the transconjugant strain 64/3xUW3114 T-10 the *E. faecalis* PAI integrated into a tRNA*lys* gene. Besides, the integration site of the *E. faecalis* PAI in *E. faecium* is distinct from the region where the esp_{Efm} PAI is located. The homology between the integration site of the *E. faecalis* PAI in *E. faecalis* and *E. faecium* presents the possibility that larger regions act as the recognition sequences for PAI integration, although the 10 bp sequence (*attP*-like) should be the effective site for homologous recombination, since it resulted in DR (*attL*- and *attR*-like) at both ends after PAI chromosomal integration.

It is known that due to the short stretches of DNA sequence similarity necessary to initiate recombination events, circular DNA molecules are more likely to be transferred and acquired, making them the most promiscuous recombinogenic states of all DNA [167]. On the other hand circularized intermediates of other PAIs have been seen and associated with their transfer [84, 99, 118, 177]. These observations prompted us to test for the excision of the PAI from the chromosome and for the formation of circular intermediates. Besides confirming that the PAI can precisely excise and circularize (only observed in the donor strain UW3114), imprecise excision and circularization were also detected (Fig. 3.2) apparently due to homology between internal regions of the PAI and flanking chromosomal regions. The circular intermediates of the PAI lacking the integrase and excisionase genes might be unable

to integrate/excise in a recipient strain. The fact that precise excision and circularization could only be detected in the donor strains but not in the reference strain MMH594 might explain why previous attempts to transfer the entire PAI element from this strain have not been successful [22]. It seems evident that the horizontal transfer event reported here resembles that of ICEs.

Esp protein was detected on the surface of all PAI-positive strains. Although FACS analysis revealed a lower *esp* expression in the *E. faecium* transconjugant than in *E. faecalis* (Figure 4.16), it is likely that the expression of genes encoded within the PAI are affected by regulatory factors inherent to the cell or species-specific. *esp* expression was diminished when cells were grown at 21 ℃, as previously described for esp_{Efm} in *E. faecium* [180]. Temperature-dependent regulation of expression is an ecological feature for regulation of pathogenicity factors also observed in other pathogens that alternate between an environmental reservoir and a mammalian host [180]. These results show that the temperature-dependent expression of *esp* is also a feature of *E. faecalis* (Figure 4.16). The acquisition of the *E. faecalis* PAI increased *in vitro* biofilm formation in *E. faecalis*. However, the acquisition of *E. faecalis* PAI was not sufficient to develop a biofilm forming phenotype in *E. faecium*. The nearly absent biofilm formation of the recipient strain, together with the low Esp on the cell surface, might explain the low biofilm formation in the *E. faecium* PAI-positive strain. Additionally, it is well-known that *E. faecium* has low biofilm forming capacity compared to *E. faecalis* [94, 133] and it has been demonstrated that although Esp can increase the biofilm forming capacity in *E. faecium* and *E. faecalis* [161, 170, 180, 56], it is neither sufficient nor absolutely necessary for biofilm formation [77, 124].

The cytolysin/haemolysin *cyl* operon encodes a bacterial toxin expressed by some strains of *E. faecalis* which in addition to mediating lysis of erythrocytes, also possesses antibacterial activity toward a broad range of Gram-positive bacteria [42]. The *cyl* operon is either encoded within large, pheromone-responsive plasmids or on the chromosome within the *E. faecalis* PAI [reviewed in 41]. The PCR results showed that the complete and intact *cyl* operon was present in the transferred *E. faecalis* PAI element. However, a strong (beta-) haemolytic activity could only be detected in *E. faecalis* strains. This is the first description of transfer of the *cyl* operon and *cyl* genes to *E. faecium*. Previous studies have reported complete absence of *cyl* genes (n=271) among *E. faecium* [183] as well as absence of both *cyl* genes and haemolytic activity (n=21) [48]. Vancanneyt et al have reported beta-haemolytic activity in 5 *E. faecium* isolates (n=78) but no link could be established between haemolytic activity and gene presence [181]. Several bacteriocins have been described in *E. faecium*: enterocin A, enterocin I, enterocin P, enterocin L50A/L50B and enterocin B [30]; however, none of these bacteriocines and/or the strains producing them possessed haemolytic activity [6, 36, 18, 19, 14]. It remains unclear what prevents the expression or activity of the *E. faecalis* cytolysin/haemolysin in *E. fae-*

cium, but at least it is clear that haemolytic activity has yet been rarely observed in *E. faecium*. The transfer of the PAI and the expression of pathogenicity-associated factors encoded within it raised the question of whether the pathogenicity of the *E. faecalis* isogenic strains carrying and lacking the PAI would be different. The bacteraemia and peritonitis mouse models used in this study have been demonstrated to be suitable in similar settings to evaluate the role of biofilm associated factors in enterococcal pathogenesis [66, 166]. However no significant difference between isogenic PAI-positive and PAI-negative *E. faecalis* strains was observed (Figure 4.20). The supposed increase in pathogenicity in PAI-positive transconjugants could have been compensated by the impaired growth observed in the *E. faecalis* PAI-positive transconjugant (Fig. 4.21). It has been suggested that larger bacterial genome sizes reduce the bacterial growth rate [174] and delayed growth or diminished fitness can compensate the expression of virulence factors of the transconjugant strains during infection. The recipient and transconjugant strains have a different genomic content, consisting of an additional ca. 66 kb plasmid and a ca. 200 kb PAI. This corresponds to almost 10% of the genome size (the chromosome size of OG1RF is ca. 2.74 Mb). However, enterococci exhibit virulence through mechanisms such as adhesion and biofilm formation, translocation through eukaryotic cell layers, or release of proinflammatory molecules and the absence of an observable effect in the models used does not exclude the possibility of an increased pathogenic potential of the PAI-positive transconjugants.

5.4 pLG2 Plasmid sequencing

pLG2, the *erm*(B)-plasmid transferred along with the PAI, carries all the elements necessary for replication including repR-pLG2 and oriR and segregation locus (PrgP-PrgO). Although a replicase and an origin of replication homologous to pEF1 are present in pLG2, no relaxase could be identified, as has also been described in other pheromone-responsive plasmids (i.e. pAD1, pCF10 and pAM373). Ruiz-Barba *et al.* suggest that pEF1 replicates using the *oriR* and *repR* using the θ-type replication mechanism [127], which could also be true for pLG2 given the homology of its replicase to that of pEF1. However, the mechanism of replication of pheromone-responsive plasmids is still unknown [186]. The PrgP-PrgO partition system of pLG2 (segE locus) constitutes a putative ParAB-like partition system [86] and is putatively involved in replication as it has been observed in pCF10 and pRE25 [62, 140]. The two genes are upstream and inverted with respect to *repR*-pLG2 and constitute another characteristic of pheromone-responsive plasmids. A similar organization of these partition protein genes, normally known as *repBC*, has been previously described in pCF10, pAD1, pPD1, pAM373 and pRE25 [62]. The conjugative transfer of Gram-positive plasmids requires elements that provide con-

tact between the mating cells, the formation of a canal to cross the cell wall and the transfer of the plasmid DNA. Homologs to all these elements were found in the DNA sequence of pLG2. One of the mobilization-related regions of pLG2 is homologous to that of bacteriocin-encoding plasmid pEF1 and the other to that of the RepA_N- family plasmid pAD1. Meanwhile, the replicase gene identified was a RepR, which is unrelated to the RepA_N- plasmid family. This suggests a modular composition of pLG2, which is a common trait of enterococcal and other plasmids [185, 78]. Phylogenetic analyses have shown that the *repA* replication gene and *repBC* loci are separable modules that do not need to be closely associated [186]. A recent report of Manson *et al.* describes that pTEF1 and pTEF2 plasmid transfer functions (i.e. *oriT*, relaxase and TraG) were essential for the transfer of chromosomal DNA among *E. faecalis* and its integration into the recipient's chromosome was recA dependent. BLAST comparisons revealed that pLG2 is different from pTEF1 and pTEF2. However, the mobilization region bearing the *oriT* in pLG2, as well as that of pTEF1 is homologous to the oriT region of pAD1. Despite this congruence, they contain different replication regions and neither a relaxase nor TraG were detected in pLG2 sequences. Although not formally proven in the present study by functional knockout experiments, it seems that the pLG2 plasmid resembling conjugative *E. faecalis* pheromone plasmids can support horizontal PAI transfer by providing the conjugative modules necessary for cell-to-cell transfer.

6. Conclusions

The horizontal transferability of enterococcal virulence associated factors might be responsible for an increased pathogenicity and spreading capacity of some enterococcal strains and clonal types.

The hyl_{Efm} gene is part of a highly conserved genetic structure that was mostly plasmid located among a wide set of clinical *Enterococcus faecium* isolates. hyl_{Efm} megaplasmids are widely distributed among clinical associated *E. faecium* and seem to be restricted to this species. Co-localization of hyl_{Efm} with metabolic and resistance genes (e.g. antibiotic, metal) on the same conjugative plasmid suggested co-selection of potential pathogenicity factors under the selective conditions imposed within the clinical environment, which might explain the rapid adaptation and increased success of hospital associated subpopulations of *E. faecium*.

The *E. faecalis* PAI can precisely excise, form circular intermediates and transfer intra- and interspecies into the chromosome of *E. faecalis* and *E. faecium*, integrating site specifically. The pheromone-responsive conjugative plasmid pLG2, transferred along with the PAI could have provided the mobilization machinery necessary for the horizontal transfer of this element. Acquisition of the *E. faecalis* PAI caused the expression of several pathogenicity-associated factors in the transconjugants such as Esp on the surface of *E. faecium* and *E. faecalis*. In *E. faecalis* PAI-positive transconjugants, cytolytic activity and biofilm forming capacity were enhanced. However, results of mouse bacteraemia and peritonitis models did not reveal significant differences in pathogenicity and survival *in vivo* of the PAI-positive *E. faecalis* strains.

The horizontal transfer of mobile elements *in vitro* might differ from the *in vivo* situation as observed in two scenarios thaare not observed in natural isolates: a. The presence of the *E. faecalis* PAI into *E. faecium* and b. The spread of hyl_{Efm} plasmids among non clinical strains. Antibiotic usage, can induce the horizontal transfer of pathogenicity traits triggering recombination or imposing a selective pressure for antibiotic resistance resulting either in the direct transfer of elements co-localized on the same element (hyl_{Efm} plasmids) or in the mobilization in trans (pLG2- *E. faecalis* PAI).

A. Appendix

Primer name	Sequence	Reference Sequence	Reference
***Enterococcus faecalis* pathogenicity island (PAI) investigation**			
esp-TIM1	CTTTGATTCTTGGTTGTCGGATAC	$gb\|AF454824.1\|$	[183]
esp-TIM2	TCCAACTACCACGGTTTGTTTATC	$gb\|AF454824.1\|$	183
ermB1	AGCCATGCGTCTGACATCTAT	$gb\|M11180.2\|$	145
ermB2	TGCTCATAAGTAACGGTACT	$gb\|M11180.2\|$	145
PAI164	ATGCCATGTTCAGCGAAGTTGCCAATTATC	$gb\|AE016830.1\|$	141
PAI167	GCTGATTTATTATGGTTCTCAGCAATCGCC	$gb\|AE016830.1\|$	141
PAI164Efm	GGCTAAGCCTTCTTGTCTTTTATCGTTAAG	$gb\|ABSW01000168.1\|$	This study
PAI167Efm	TAGTCAATCTAAGCGGGAATGTTGTTTT	$gb\|ABSW01000168.1\|$	This study
EF2	CCAAAAAGCAACTTTCAACC	$gb\|AF454824.1\|$	111
ER1	ATTCAAGAATGGCTGGGAC	$gb\|AF454824.1\|$	111
PAI164nested	TTATACAACGGGGGCATAGC	$gb\|AE016830.1\|$	This study
PAI167 UW3114	AAACGTCCTAAGACGCCGACAGAATAC	$gb\|AE016830.1\|$	This study
PAI167nested	GATTCGAACCCTAGACCCTCT	$gb\|AE016830.1\|$	This study
Genomic walking outwards of *Enterococcus faecalis* PAI			
TSP1 5'	GAAGATGGACGGTTGATGAAGCCTC	$gb\|AF454824.1\|$	This study
TSP2 5'	GGCTGGGACATGCATCGTATTCG	$gb\|AF454824.1\|$	This study
TSP3 5'	CGTGCAGCAGAAGCATTAGAAAACGC	$gb\|AF454824.1\|$	This study
TSP1 3'	GCGGAATTCTGGTATTGAGC	$gb\|AF454824.1\|$	This study
TSP2 3'	TGAACTTGCCAAATCAGTGG	$gb\|AF454824.1\|$	This study
TSP3 3'	CCTACCAATTGCCAAGGAAAT	$gb\|AF454824.1\|$	This study
***Enterococcus faecium* hyl_{Efm} genomic island (GI) investigation**			
hyl1	GAGTAGAGGAATATCTTAGC	AAAK03000042.1	120
hyl2	AGGCTCCAATTCTGT	AAAK03000042.1	120
hylGI1-F	CATTTAGAAGGCGAGATGTCTTCAGATAA	HM565216	This study
hylGI1-R	TCTCAATGATCGAGGTTTCTTCAATGTAT	HM565216	This study
hylGI2-F	TGCCTTCCACGTTTTTTAGCTCAAGGAGGAGAG	HM565216	This study
hylGI2-R	GGCCGTTGATAGACAGAAGCCATTTCCTGC	HM565216	This study
hylGI3a-F	ACAGTTAGAAGAAGTCTGGAAACCGGTAA	HM565216	This study
hylGI3a-R	GGCCGTTGATAGACAGAAGCCATTTCCTGC	HM565216	This study
hylGI3b-F	CAATTACCGTTCTTTTGGACTG	HM565216	This study
hylGI3b-F	ATCGTTTCAACAGAGCTTGC	HM565216	This study
hylGI3c-F	ACGGTTGCTAATACTCTTCCAC	HM565216	This study
hylGI3c-R	ATTGGAGATGCTGGATACGG	HM565216	This study
hylGI3d-F	TGTGCGACTTCATTTACGATACGGAC	HM565216	This study
hylGI3d-R	AAGGTCAAGTACCTAGTGATGGACATGAA	HM565216	This study
Hyl PAI-4F	TCCGCATTTGGAAAGACCGTTTTAAGCAACTGAC	AAAK03000042.1	This study
Hyl PAI-4R	TTCGACAAGAATTGACGCAGCTTTATAACCCGC	AAAK03000042.1	This study
vanA1	TCTGCAATAGAGATAGCCGC	M97297.1	74
vanA2	GGAGTAGCTATCCCAGCATT	M97297.1	74
vanB1	CATCGCCGTCCCCGAATTTCAAA	AY665551.1	74
vanB2	GATGCGGAAGATACCGTGGCT	AY665551.1	74
Genomic walking outwards of hyl_{Efm} genomic island (GI)			
TSP1hylGI 3'	GAAAACACCGAACTCACGAATACGGC	AAAK03000114.1	This study
TSP2hylGI 3'	GTGGCTCCCATGTCGTGGAATC	AAAK03000114.1	This study
TSP3hylGI 3'	TCCGCATTTGGAAAGACCGTTTTAAGCAACTGAC	AAAK03000114.1	This study
TSP1hylGI 5'	CCCGCTTATTTAGGACGGGCCTTC	AAAK03000042.1	This study
TSP2hylGI 5'	TATTCAGCCCCTCAGTGACGCCGTTTG	AAAK03000042.1	This study

TSP3hylGI 5'	CCCCCGCTGAGGTTGAGGAATATGG	AAAK03000042.1	This study
Sequencing primers hyl_{Efm} genomic island (GI)			
HylPAI3 seq1 F	CAATTACCGTTCTTTTGGACTG		This study
HylPAI3 seq2 R	TGCGTAAAGGTGAAGCAGAAC		This study
HylPAI3 seq1 F	CAATTACCGTTCTTTTGGACTG		This study
HylPAI3 seq2 F	ACGGTTGCTAATACTCTTCCAC		This study
HylPAI3 seq2 R	TGCGTAAAGGTGAAGCAGAAC		This study
HylPAI3 seq 3 F	AGTGCCCGACCAGAAACATTGC		This study
HylPAI3 seq2 R	TGCGTAAAGGTGAAGCAGAAC		This study
HylPAI3 seq2 R	TGCGTAAAGGTGAAGCAGAAC		This study
HylPAI3 seq 3 F	AGTGCCCGACCAGAAACATTGC		This study
HylPAI3 seq1 R	ACTTTTATAACTTTCGGGCACG		This study
HylPAI3 seq 3 F	AGTGCCCGACCAGAAACATTGC		This study
ctg 625DO del F	ACACCTTGATAGGTGGGCTG	AAAK03000042.1	This study
ctg 625DO del R	ATTGCCACTCTTGCAAATCC	AAAK03000042.1	This study
Hyl PAI gap F	TGGCGAAACTTATGGATTGCCAG	AAAK03000042.1	This study
Hyl PAI gap R	TCCATGTCCCGCTAGAGCTTC	AAAK03000042.1	This study
Backbone pLG1			
res-p-hylF	CGTTTAATGGGGTAACTGACCCTGC	HM565168	This study
res-p-hylR	ACGGTTTTTCTTGTAACTCCTGCGT	HM565169	This study
rep-pUW2774F	AGTCGAAAATATCCAGAACCTG	HM565183	This study
rep-pUW2774R	AACCAGTCTTTCGTAATAGTGC	HM565184	This study
mobC-pUW2774F	ATCAATCGTATACTCTGAATACCCCTCA	HM565181	This study
mobC-pUW2774R	AAGGGGAATTTTCGTTTTGCCCCAT	HM565182	This study
rel-pUW2774F	GTGGACTGGAAAAAATGCAAG	HM565192	This study
rel-pUW2774R	TTTTATCGTTCTCAGGAAGTGG	HM565193	This study
repB-pUW2774F	GTTTGAATCAGCAGTTAATGAAGG	HM565222	This study
repB-pUW2774R	CCAATCATCATACATTTCAAAGGAC	HM565223	This study
MazE-pUW2774F	GGGAAAAGAACTAAAGGTTAGAAAG	HM565183	This study
MazE-pUW2774R	CCATTCTTCTATTATCGTTTCAAGG	HM565184	This study

Table A. 1: Primers used in this study for investigation of the *Enterococcus faecalis* PAI and *E. faecium* genomic island (GI). $gb|AF454824.1|$ is *E. faecalis* pathogenicity island (PAI), $gb|AE016830.1|$ is *E. faecalis* V583, $gb|ABSW01000168.1|$ is from *E. faecium* strain U0317, $AAAK03000042.1$ is *E. faecium* contig 625, $HM565216$ is plasmid pLG1 contig 65 and $AY665551.1$ is *E. faecalis vanB* gene.

Primer name	Primer sequence	Amplicon	Location
long template PCR		(bp)	
PAI164	ATGCCATGTTCAGCGAAGTTGCCAATTATC	1548	C:
1 PAI R	GGAAGATGGACGGTTGATGAAGCCTCAATATG		427
2a PAI F	CAGTTGTCGAATACGATGCATGTCCCAGCC	9674	240
2a PAI R	AAACCAAAGGAACCGAAACGGAAAAACTTAGCATGG		9913
2b PAI F	TTTAACCAGCCATGCTAAGTTTTTCCGTTTCGGTTC	6066	9869:
2b PAI R	TTTGAAATAATCTCCAACTTTTCCCCCGTTCCACAC		15934
2c PAI F	AACCATAAAAAGGAACGGAGGGAGCACAACAAAAGG	7697	14033:
2c PAI R	ACTTGCAGTGTGACTGTCTGTCGTAACTTCACC		21999
3a PAI F	CTCGTCCGTAACGATCTGTTTTATCGCCCTTATC	11645	21566:
3a PAI R	TCAAGTCCGTACAACAGGCACTTTCTTTATCAAGC		32079
3b PAI F	GAAGGCCGTTGCCAATTTTGCATTAGCTTGC	11411	31320:
3b PAI R	TCCTAAGCCTATGGTAAAACATGCTGGAGTTGTCTC		42730
4a PAI F	CAAGGTAGTGGAGATGTTCAGGCTGAGACAACAC	11079	42395:
4a PAI R	CGGATGTTACTTCTGCTGGACTTAAAACAATCCC		53473
4b PAI F	GGGATTGTTTTAAGTCCAGCAGAAGTAACATCCG	11561	53440:
4b PAI R	ACGCCAAGCACAAGGGATAAAGATTGCGAAAG		65000
5a PAI F	GGACGACCTTTATAGACGCCGTTTGCTTTCG	10629	64944:
5a PAI R	AGTCCCCTTTTTCTGCCATGACACCAGTTAAAATC		75572
5b PAI F	GCTGTGGTCAAGATAGATGGGAAAGAGATTGAGCG	11431	74158:
5b PAI R	GGATCTGAACCGTCTTGTGTCATAGTGTGCCAG		85588
6a PAI F	TGTAGCATACTGGCACACTATGACACAAGACGG	9115	85547:
6a PAI R	CGTGCCCCTAATTACCATAGAGATAGTCGCGTTG		94661
6b PAI F	TGGTAAACGCTGCTCCTGAAATGAAGAGTTTGAC	8432	93984:
6b PAI R	AGGTTTGATACGCAACTACCTTTCCCAACTGACG		102421
7a PAI F	TTTTGGGACAGGAACGCTATCAGTTAACGATTGC	10821	101954:
7a PAI R	CCTGCGGTCAAGCACAGTTGCCTTATCTTAG		113046
7b PAI F	ATTAAAGTCAAAAGAGACTGTTACTTGTGCGCCCTG	13858	113008:
7b PAI R	TCAGCAAACTAAGATAAGGCAACTGTGCTTGACC		126865
8a PAI F	TGCTTTAGTGGGTCGTACTAACGGAACAATAG	11008	125344:
8a PAI R	CAAACAACACGTCGTCGATCTTTACCTTG		136351
8b PAI F	CACCAATGCACATAATCAAACAATTCTAGGCGTAG	11048	135337:
8b PAI R	GTGGACAAGCACAGTCACAATTAGAAGCAATG		146384
9 PAI F	CATCATTTCTTCAGCAAATTGGTTGGCACGC	8298	146272:
PAI167R	ATGTTGGTTGAAAGTTGCTTTTTGGCAAAC		C
Regular PCR			
PAIefs-11F	AGAATTGTCTTTGGGCTTCTC	378	
PAIefs-11R	GCCGAATGTTGCTAAAGTTAC		
PAIefs-12F	GGAAAATGTTTAAAAGCCCCC	486	
PAIefs-12R	GGCAACGTGAGAGTAACAG		
PAIefs-21F	GCCATTAAAAAGTAAAGAGCCG	500	
PAIefs-21R	CTTGATATGCGCGATAAAGTTG		
PAIefs-55F	AGGATTGCTTACGGTTGATGG	357	
PAIefs-55R	TTTGCTTTCTCTGTTTGCTCAC		
PAIefs-58F	GGTGTTGTTCCTGTGGTTAG	536	
PAIefs-58R	TTAGGAGTCGTCCCTGTTATC		
PAIefs-65F	TTTTCCAGCTAAAGAACCACC	446	
PAIefs-65R	GTAAATACCACGATGAAGTTGC		
PAIefs-70F	CTTGGGATAGTGAACAAGATGG	377	
PAIefs-70R	CAAGGACTGCGCTATGAAG		
PAIefs-84F	GCCGATCCTACATATACAAACG	417	
PAIefs-84R	TCCAATAGTTGATCATACTGCG		
PAIefs-87F	GTTATTGGCAGTGCCGTTAG	433	
PAIefs-87R	GACTTGTGAAAGTCCTCGAAG		
PAIefs-89F	AAGTATTCTAGGCAGTTTGCG	403	
PAIefs-89R	GCCACGTTCTTTAGAAATTCG		
PAIefs-91F	TTGGGACAGGAACGCTATC	487	
PAIefs-91R	GGCCCCATACTTTTGTCATC		
PAIefs-92F	GAAACAGGTAGACGCTAACG	521	
PAIefs-92R	AACGTACTTCTTTTCCCGC		

PAIefs-95F	GGAAACTGGAGGAAATGCTTG	508
PAIefs-95R	CGGTCTATCGTCCACACTAC	
PAIefs-99F	AATTTGGACTTCCAAGCGAC	530
PAIefs-99R	ACTATTTCATTCCCCAGGGC	
PAIefs-102F	GCGCATTTAAGAGTGCAAATTG	582
PAIefs-102R	TGCGGCTAGTAAGTAACCAAC	
PAIefs-108F	AATGCAAACCAGCTCTTAGAC	409
PAIefs-108R	GGTCGATGGTCAATTCATCAG	
PAIefs-111F	GTGACGACAGAAGAACAACTAC	585
PAIefs-111R	TCTCTTGAAAGGAACTGCCC	
PAIefs-115F	CCACCAATGCACATAATCAAAC	471
PAIefs-115R	GTAATGAAGCGGAGACAACG	
PAIefs-122F	TGTTTCAGGTTCAAGTTCAGC	505
PAIefs-122R	TCAACCTCAATGGAAAATGTGG	
PAIefs-128F	ACCAGAAAAACTGAAAGAGACG	548
PAIefs-128R	GACTGGAAGCATTTTGGGC	
PAIefs-13F	AAAGAGCAGCAAGTCCTAAAG	293
PAIefs-13R	TGAGAAACGGTCACAACAC	
PAIefs-14F	AAGGCAATGCTCAATCAGG	251
PAIefs-14R	CCTACGTATCTTCTTTTCGGTG	
PAIefs-15F	TATGAACGCCCAGCGATTC	202
PAIefs-15R	GGGGGACTTTACTTTCTACGAC	
PAIefs-16F	TTTAGCTGCTGGATTGCG	229
PAIefs-16R	CGTTGGTCTATCAAGCAACTTC	
PAIefs-17F	GGTGATTTTAGGACTGGTATCC	199
PAIefs-17R	TCGCCAACCTTTTATTGGG	
PAIefs-18F	TTTGCGCCAAACGGCATTC	181
PAIefs-18R	AAGAAAAGAGCGTCCACACAAG	
PAIefs-19F	AGATTACACCGAGGATGTAGC	174
PAIefs-19R	TCTTCGCTCTCACAATCAAAAG	
PAIefs-20F	TCAACTGTGTTATTTGGCGG	218
PAIefs-20R	CGTAGCTTTACCTGTAGCCTG	
PAIefs-57F	ACGTCAAACCGTATCCAAAG	179
PAIefs-57R	AGCCGTCCGTAATAAAAAGC	
PAIefs-59F	GCAGAAAAAGGGAAGAAATTACAAC	289
PAIefs-59R	CTGGAAAAATACCGAATCTTAGTTG	
PAIefs-60F	GCTTGACCAGATGACTAATGC	180
PAIefs-60R	GCAACGAACTCATCAACAAATG	
PAIefs-61F	GGATTTCCGATTGCTTCAGC	198
PAIefs-61R	AGCGACTATTACTCATCGACC	
PAIefs-62F	GAAGCCATTGACCGATTAGG	185
PAIefs-62R	TGTTCCGCAATCATTGCC	
PAIefs-66F	TCAAGCTGGTGTGGATTTTG	198
PAIefs-66R	CCACCAACGACAATTCCTG	
PAIefs-67F	GCAACTAGCTGATCCAAACC	181
PAIefs-67R	AAATGAAGTCAAGCCACCAC	
PAIefs-68F	TTGACTGGACGAATTGAAGC	222
PAIefs-68R	GACAGATGGATCTTTTCCTGC	
PAIefs-69F	GCCATACTTCCGTCAATAACG	163
PAIefs-69R	TGTCACTACCATTAATCACTGC	
PAIefs-72F	TGAAAATGGCACTCCAACAG	226
PAIefs-72R	CCGTACATCATAGGCACAATC	
PAIefs-73F	GAGTACTGGATTATTCAGGCG	212
PAIefs-73R	TTGAATGGCTTCTTCACCG	
PAIefs-74F	GGGATAAGCAGACGATTATCGG	155
PAIefs-74R	TCTTGTGCGTGGTGCTAAC	
PAIefs-76F	TGCCATAATGGGTATGAGGAC	212
PAIefs-76R	CATGCTGACCTTTAACGGTAG	
PAIefs-78F	GGTTCACAAAGCTGAAGCAG	198
PAIefs-78R	CCTCCAGCAACAGTTAATCC	
PAIefs-79F	AGAGAATGCTGACGAAAACAC	191
PAIefs-79R	GCGCTACACTTACAACAATCC	

PAIefs-80F	AGAATTGATGATCGCTTGATCC	153
PAIefs-80R	CGAAGAAATTCCAGCAGGAAC	
PAIefs-81F	CCTGTTGTTTCTATGACGCC	200
PAIefs-81R	ACCATTCCCAAATTAACTCCAG	
PAIefs-82F	ACGATTTCGATGCTGCAAC	202
PAIefs-82R	TCATCTGTATCCCAGCCAAG	
PAIefs-83F	GGAGCTGATAATGCTTGTGC	202
PAIefs-83R	AAGAATTACCTGCTGCCAAC	
PAIefs-92F	GCTTTAGTTTCACTACAATCCCC	194
PAIefs-92R	GCGTCTACCTGCTTCTTAATTAC	
PAIefs-93F	GGGAACATATGCCGGAATG	293
PAIefs-93R	CATGATGCACAATTAGCACTG	
PAIefs-94F	GGCGATGCTATTTCTCATGC	223
PAIefs-94R	CGGTTGAGCTATTTGCAACAC	
PAIefs-119F	TCATCTCCTTCTTTCCCTGC	193
PAIefs-119R	AACGACTGATTACGCTTGAC	
PAIefs-123F	CCACCGAAGTTGAGTGTATC	198
PAIefs-123R	TCGATCTTCTGCTTCTTGTTC	
PAIefs-126F	CCAAACACGACTTCTTGCTC	198
PAIefs-126R	CACGGATTCACTGCTAAAGG	
PAIefs-129F	AGCCAATGAAACGACAGAAG	214
PAIefs-129R	CGGCTCAATACCAGAATTCC	
PAIefs-128-129 F	ATCAGGTCAAACGGATCAAA	197
PAIefs-128-129 R	CTGACAGAATGGCTCGTCTT	
PAIefs-125-F	CTAGGAATACAGCGACCAATAC	597
PAIefs-125-R	CCGACTTTGCTATCGGAAC	
PAIefs-126-F	TTTTGTCCAAGTGACATCGG	628
PAIefs-126-R	TTCAGAGATGGAAACAAGAGTG	
PAIefs-128F	AGGGACAGGGTTTTCGATTT	477
PAIefs-128R	ATTTGGTTCGCCTTCCTCTT	
oxiRed-F	AACTGGTTTGAACCGTGAGG	385
oxiRed-R	TTGCATCAATGGTGACCAAG	

Table A. 2: Primers used for investigation of *Enterococcus faecalis* pathogenicity island (PAI) presence by regular PCR. Location given as in *gbAF*454824.1 *E. faecalis*PAI.

Primer name	Primer sequence	contig	location
pTEF3 like F	TTGTTCGCTAAATGCACCGT	1	1533:2182
pTEF3 like R	GCACGTACAATTCCTGCGTT		
pili F	TCCGTGCTTTCTTGTCTGTCTGCAAACGTC	21	34386:34688
pili R	ATGTACGCCTAAGGGGATCAACACACACCG		
ctg2 F	GGAACAGGTAAAGGGCATTTAACGAC	2	713:1268
ctg2 R	TGTTTACTTTGGCGTGTTTCATTGC		
ctg6 F	TGCTCGTATTATTGCTCGTTTACCG	6	1002:1658
ctg6 R	TGCCAAGTGACTGTCGTACATAAAG		
ABC-like F	TCCAGAAGTTGGTTCATCGG	4	11085:11513
ABC-like R	GAAAAGGGTGAATTTGTTGGTC		
mano F	TGGTTGCCGGACGTTTTTGGCTATTCTTGG	12	30944:31299
mano R	AACATATCACGATTAACGCCGCCTCCGC		
bleo F	CGATGCCGGAAACCGAAAATAGTCGC	12	33451:33943
bleo R	TGGCCGATATCACTACCAAACCAGACG		
ctg16 F	AAGATGCTAAAGTAGTTAGCGGAGAAC	16	451:1017
ctg16 R	CACTCACTGACTCTAAAGACTCTTCAAAC		
ctg17 F	GATCATCAATCGTATACTCTGAATACCCC	17	399:970
ctg17 R	CAATCAAATAGCTCTTGCGAAGGC		
ctg17cr F	TGAGTAATTAGTGCCGCAAAAACAGG	17	2818:3511
ctg17cr R	ATTTGGGTGAAACGGGTGTTTACG		
ctg18 F	TTTCCATGATCGATGAGCAGGAAC	18	4051:4708
ctg18 R	CGAAAAAAGGCTCTTCGACATTTAGTG		
ctg20 F	TTGCCCAACAAATGCGATCACATC	12	5278:5928
ctg20 R	ATAGCTTCATCTCCACGTAAGCCG		
ctg21 F	GGCTGATGGGGTTTTTTTTACGTTTC	21	43160:43710
ctg21 R	ACTCTATACACGGTGACCGCATAC		
ctg24 F	TCGTTCTCTGTGATGAAATTGCCTAC	12	9056:9739
ctg24 R	ACACGAGTTTTGCTACTTAACACGAG		
ctg26 F	GCGCTACAATTACTCATCGACCTG	26	366:1008
ctg26 R	ACGACAATTGATTATGAAGCCAGAGAG		
ctg29 F	GAAACTCAGGAGCACATTGTTCTAGATTC	29	801:1494
ctg29 R	GCTGAAGCAATTGGCAATCCTCTC		
ctg32 F	TTGGCATCCCTCCACAACAAAAAC	32	562:1173
ctg32 R	CATCACTTATTTTTGCAACCGCTGAAG		
ctg34 F	GCAGCTTGATTTATCTCATCTTGAACAGC	34	507:1158
ctg34 R	TACCAGAGCCAAATCCATTGCCTG		
ctg35 F	TCCAGCTACTGGAATATACTGCTGTAATC	35	996:1628
ctg35 R	ACGAAAATTTCCACTGTGTGACCG		
ctg40 F	CACTCTAACCAGTCTTTCGTAATAGTGC	21	40770:41361
ctg40 R	TACCCACCCTCGACGAAAAATCAG		
ctg67 F	AAAACCCCTAAAATGACGGCCTTG	12	28068:28714
ctg67 R	GCAACCATTGCCTTAGCAACTTTG		
ctg69 F	CTCCAGTTGATACACATCTAGCTGC	4	6705:7382
ctg69 R	GAGCTTATTTTGGAGAAAAGGATGCAC		
ctg70 F	ATTCTGATGGCAATAAGGGGCAG	70	3005:3681
ctg70 R	CCACTGATGACGGTGATAACATTTTTC		
ctg71 F	GGGGAGGGAGAAGTTAAAGAAAATTCAG	3	1457:2017
ctg71 R	ATTATGGCAAGGGGAGGAGGTTG		
ctg75 F	TTTTACGTCCACTAGCCAACTGTC	3	11232:11902
ctg75 R	CAGGATTTTACCACCAGCTTTTGC		
ctg76 F	ACCACGAATCCTATTATAGAGACATGGAC	76	4367:4960
ctg76 R	GGACCAAGCCCTCTGATTTTCTTTTC		
ctg78n F	TGCGGGCACAACATATAGAGTAGCTTACG	78	621:1220
ctg78n R	TCACCTTCTCCGCCTCCTTCTTCGTTAG		
ctg87 F	CTTAGGTTCGGGAAGTTTAGGCG	87	3567:4161
ctg87 R	AGCCGACATCATTTTCTTCAGCG		
ctg91 F	TCTTGCTCAATGCTTTACGGTTAAGAC	91	3711:4378
ctg91 R	GCCATGATCCATTTCACTGTGATCC		
ctg92 F	CTGTTGGCAAACCACAAGTGACC	92	134:708
ctg92 R	ATCACCTACAAAGGCGACTGCTC		

vanA1	TCTGCAATAGAGATAGCCGC	6	6309:6685
vanA2	GGAGTAGCTATCCCAGCATT		
hyl1	GAGTAGAGGAATATCTTAGC	65	12765:13426
hyl2	AGGCTCCAATTCTGT		

Table A. 3: Primers used for synthesis of 31 probes to confirm plasmid location of pLG1 contigs by Southern hybridization. The putative function of the ORF covered by the probe is indicated, some probes cover more than one putative ORF. All probes hybridized to the plasmid pLG1 band resolved by S1 nuclease Pulse field gel electrophoresis (PFGE) treatment.

Primer name	Primer sequence	Product size (bp)	Result
lysteria gap F	GGAGATTTTGTGTTGATTCCCCCAC	1736	3000
lysteria gap R	AATTGTATCCTCAGGCAATGCCG		
DO658 gap1F	CATACTTCTCCCTGGATAACTTCCC	978	+
DO658 gap1R	CAAACAAGATTTGCATCATATCCATT		
DO658 gap2F	CTTCATTAAAAGGAGCTTGACCGAT	357	+
DO658 gap2R	TTGTCCTTGTTGTCCAACTTCAAAT		
DO658 gap3F	AGTTTTAGTTTTGGGGCTGTTTTCA	1909	+
DO658 gap3R	CAATCCCCTCTGATAATTCGGATAC		
DO625 gap1F	ACTATCGTTTTGCAACGAGGACC	300	-
DO625 gap1R	AGTGTCCGTATCGTAAATGAAGTCG		
hylPAI3c-ISF	CGAGGACCAACTGGACCGTAGGAGC	2576	+
hylPAI3c-ISR	ACCACCTGCAACAGTGGAGTGGGAA		
DO614 gap1F	CCGTCAACTCATATGCGAGTATTTC	643	2000
DO614 gap1R	TCTCTTTTGCCTACTGTTGATCGTC		
DO590 gap1F	GAGACTTTATCGGAAGAAGCGAATC	790	-
DO590 gap1R	CATGTTTTATTGTGCTGGTTCATCA		
DO590 gap2F	GCATTTTCCCAATACACTCAGTTTG	880	+
DO590 gap2R	ATAGGAGGCTTGGTTCCTAGTGTTG		
DO590 gap3F	TTGTAACCGGTCAATTTCATAATGG	826	+
DO590 gap3R	CAAGGAACTGACATTGGCATAAAAG		
DO590 gap4F	GCAAAGTACCGTTTCCAGACATCTA	853	+
DO590 gap4R	CGTCTTTTTCTTCTGAAAGGTCTCG		
DO590 gap5F	AGAACTTCCCGAAACGTTAGATGAC	465	-
DO590 gap5R	ACGTAAATGGAAAGTTTTGTCTCGC		
DO632 gap1F	CTACACGTGGCTTTCCAGCTAAGTA	373	+
DO632 gap1R	CACGGTGTCGATTTTTATGACTTTC		
DO632 gap2F	CCACTTTCGTGAAGTTTAACAATGG	848	+
DO632 gap2R	AAGTCCCAAATGGAGCAACTGTATT		
DO632 gap3F	GACCTAAGGTTGTTTTTGTCGATGA	1448	+
DO632 gap3R	CAATCAAAAGAAAATTGCGAATCAC		
DO649 gap1F	CAATTGTCCAAATTTATCGTCCAAA	814	+
DO649 gap1R	TGAGACAATTATGACAAACGACAGC		
DO649 gap2F	TTGTAGATCCTCTACTTTCACGTTGTTTT	866	+
DO649 gap2R	GCAACTCATTAGCTAGACAAACTACAAGC		
pVEF2 gap F	ATAGCCGCGGCAGTGCCATTGATCT	949	-
pVEF2 gap R	ACTTCACACTTTCTTGGCGAACAGGA		

Table A. 4: Primers used for gap closure of plasmid contigs during pLG1 sequencing. 17 PCR reactions were done, of which 12 were positive and allowed linking of 15 gaps (primerDO632gap3 linked 3 contigs).

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